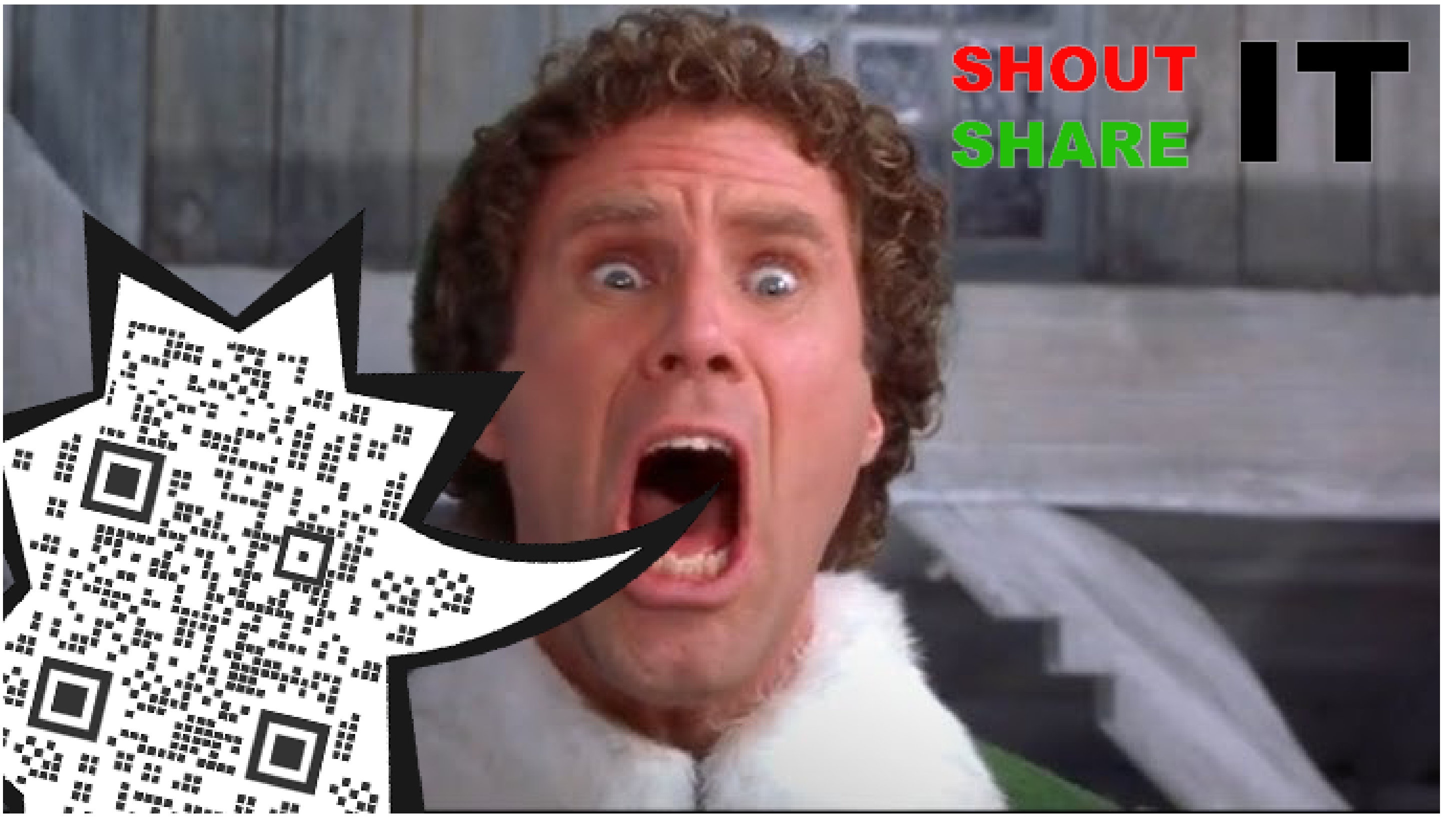
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THE
BIOLOGICAL BULLETIN

PUBLISHED BY
THE MARINE BIOLOGICAL LABORATORY

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VOLUME 105
AUGUST TO DECEMBER, 1953

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ERRATUM

On page 162 of Volume 105 (August, 1953 issue) in the article by H. L. Rosenthal, the first two sentences of the final paragraph should read as follows:

The data presented in Table II show the changes that occur in the nitrogen, calcium and phosphorus content of the dry fat-free tissue during growth and development of the Lebistes embryo. It is apparent that the total protein remains essentially constant for both the lordotic and normal strains although the values obtained for the lordotic strain are somewhat smaller due to the smaller size of the embryo.

Vol. 105, No. 1 August, 1953

THE BIOLOGICAL BULLETIN

PUBLISHED BY THE MARINE BIOLOGICAL LABORATORY

THE MARINE BIOLOGICAL LABORATORY

FIFTY-FIFTH REPORT, FOR THE YEAR 1952—SIXTY-FIFTH YEAR

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II. ACT OF INCORPORATION

No. 3170

COMMONWEALTH OF MASSACHUSETTS

Be It Known, That whereas Alpheus Hyatt, William Sanford Stevens, William T. Sedgwick, Edward G. Gardiner, Susan Minns, Charles Sedgwick Minot, Samuel Wells, William G. Farlow, Anna D. Phillips, and B. H. Van Vleck have associated themselves with the intention of forming a Corporation under the name of the Marine Biological Laboratory, for the purpose of establishing and maintaining a laboratory or station for scientific study and investigation, and a school for instruction in biology and natural history, and have complied with the provisions of the statutes of this Commonwealth in such case made and provided, as appears from the certificate of the President, Treasurer, and Trustees of said Corporation, duly approved by the Commissioner of Corporations, and recorded in this office;

Now, therefore, I, HENRY B. PIERCE, Secretary of the Commonwealth of Massachusetts, *do hereby certify* that said A. Hyatt, W. S. Stevens, W. T. Sedgwick, E. G. Gardiner, S. Minns, C. S. Minot, S. Wells, W. G. Farlow, A. D. Phillips, and B. H. Van Vleck, their associates and successors, are legally organized and established as, and are hereby made, an existing Corporation, under the name of the MARINE BIOLOGICAL LABORATORY, with the powers, rights, and privileges, and subject to the limitations, duties, and restrictions, which by law appertain thereto.

Witness my official signature hereunto subscribed, and the seal of the Commonwealth of Massachusetts hereunto affixed, this twentieth day of March, in the year of our Lord One Thousand Eight Hundred and Eighty-Eight.

[SEAL]

HENRY B. PIERCE,
Secretary of the Commonwealth.

III. BY-LAWS OF THE CORPORATION OF THE MARINE BIOLOGICAL LABORATORY

I. The members of the Corporation shall consist of persons elected by the Board of Trustees.

II. The officers of the Corporation shall consist of a President, Vice President, Director, Treasurer, and Clerk.

III. The Annual Meeting of the members shall be held on the second Tuesday in August in each year, at the Laboratory in Woods Hole, Massachusetts, at 11:00 A.M., and at such meeting the members shall choose by ballot a Treasurer and a Clerk to serve one year, and eight Trustees to serve four years, and shall transact such other business as may properly come before the meeting. Special meetings of the members may be called by the Trustees to be held at such time and place as may be designated.

IV. Twenty-five members shall constitute a quorum at any meeting.

V. Any member in good standing may vote at any meeting, either in person or by proxy duly executed.

VI. Inasmuch as the time and place of the Annual Meeting of members are fixed by these By-laws, no notice of the Annual Meeting need be given. Notice of any special meeting of members, however, shall be given by the Clerk by mailing notice of the time and place and purpose of such meeting, at least fifteen (15) days before such meeting, to each member at his or her address as shown on the records of the Corporation.

VII. The Annual Meeting of the Trustees shall be held on the second Tuesday in August in each year, at the Laboratory in Woods Hole, Mass., at 9:00 A.M. Special meetings of the Trustees shall be called by the President, or by any seven Trustees, to be held at such time and place as may be designated, and the Secretary shall give notice thereof by written or printed notice, mailed to each Trustee at his address as shown on the records of the Corporation, at least one (1) week before the meeting. At such special meeting only matters stated in the notice shall be considered. Seven Trustees of those eligible to vote shall constitute a quorum for the transaction of business at any meeting.

VIII. There shall be three groups of Trustees:

(A) Thirty-two Trustees chosen by the Corporation, divided into four classes, each to serve four years. After having served two terms of four years each, Trustees are ineligible for re-election until a year has elapsed. In addition, there shall be two groups of Trustees as follows:

(B) Trustees ex officio, who shall be the President and Vice President of the Corporation, the Director of the Laboratory, the Associate Director, the Treasurer, and the Clerk;

(C) Trustees Emeriti, who shall be elected from *present* or *former* Trustees by the Corporation. Any regular Trustee who has attained the age of seventy years shall continue to serve as Trustee until the next Annual Meeting of the Corporation, whereupon his office as regular Trustee shall become vacant and be filled by election by the Corporation and he shall become eligible for election as Trustee *Emeritus* for life. The Trustees *ex officio* and *Emeritus* shall have all the rights of the Trustees except that Trustees *Emeritus* shall not have the right to vote.

The Trustees and officers shall hold their respective offices until their successors are chosen and have qualified in their stead.

IX. The Trustees shall have the control and management of the affairs of the Corporation; they shall elect a President of the Corporation who shall also be Chairman of the Board of Trustees and who shall be elected for a term of five years and shall serve until his successor is selected and qualified; and shall also elect a Vice President of the Corporation who shall also be the Vice Chairman of the Board of Trustees and who shall be selected for a term of five years and shall serve until his successor is selected and qualified; they shall appoint a Director of the Laboratory; and they may choose such other officers and agents as they may think best; they may fix the compensation and define the duties of all the officers and agents; and may remove them, or any of them, except those chosen by the members, at any time; they may fill vacancies occurring in any manner in their own number or in any of the offices. The Board of Trustees shall have the power to choose an Executive Committee from their own number, and to delegate to such Committee such of their own powers as they may deem expedient. They shall from time to time elect members to the Corporation upon such terms and conditions as they may think best.

X. Any person interested in the Laboratory may be elected by the Trustees to a group to be known as Associates of the Marine Biological Laboratory.

XI. The consent of every Trustee shall be necessary to dissolution of the Marine Biological Laboratory. In case of dissolution, the property shall be disposed of in such manner and upon such terms as shall be determined by the affirmative vote of two-thirds of the Board of Trustees.

IV. REPORT OF THE TREASURER

The Treasurer's Report for 1952 is this year presented in a slightly different form. A condensation of the Auditor's Income and Expense statements for the past five years is submitted, which I hope you will all study carefully. It points out the need of the Laboratory for additional income and the importance of continued efforts to be put forth to secure special donations each year, as it is obvious that it is these special donations which have permitted the Laboratory to continue functioning.

In addition, the Balance Sheet, Condensed Income and Expense Statements and Statement of Surplus as reported in the annual audit by the firm of Seamans, Stetson and Tuttle of Boston are included, as well as a summary of the cash transactions passing through our various banking accounts.

There has been a little change in the market value of the securities in the Endowment Funds during the past year. The market value on January 1, 1953, was $1,166,063.74 in comparison to the market value shown January 1, 1952 of $1,108,645.75. The average yield on the securities was 4.55% on book value, 3.78% on market value. The investments and percentages in the Endowment Funds as of January 1, 1953, were:

	Book Value	%	Market Value	%
Bonds				
U. S. Government	$344,004.81	35.45	$ 333,155.27	28.57
Railroad	54,613.02	5.63	58,545.00	5.02
Public Utility	90,192.50	9.29	84,737.50	7.27
Industrial	61,518.75	6.34	60.687.50	5.20
Total Bonds	550,329.08	56.71	537,125.27	46.06
Preferred Stocks	127,173.50	13.11	120,632.75	10.35
Common Stocks	290.104.71	29.89	505,494.38	43.35
Total Securities	967,607.29		1,163,252.40	
Principal Cash	2,811.34	.29	2,811.34	.24
Totals	$970,418.63	100.	$1,166,063.74	100.

MARINE BIOLOGICAL LABORATORY BALANCE SHEET, DECEMBER 31, 1952

Assets

Endowment Assets and Equities:		
Securities and Cash in Hands of the Hanover Bank, New York, Trustee	$ 970,418.63	
Securities and Cash in Minor Funds	21,198.88	$ 991,617.51
Plant Assets:		
Land	$ 113,626.38	
Buildings	1,488,930.19	
Equipment	333,012.06	
Library	431,834.91	
	$2,367,403.54	
Less Reserve for Depreciation	850,842.36	$1,516.561.18

Current Assets:			
Cash		$ 35,988.19	
Mortgage Note Receivable		2,350.00	
Accounts Receivable		18,462.80	
Inventories:			
Supply Department	57,123.96		
Biological Bulletin	16,707.09	73,831.05	
Investments:			
Devil's Lane Property	36,881.56		
Stock in Gen. Biol. Supply House, Inc.	12,700.00		
Other Investment Securities	47,101.25		
Retirement Fund	22,142.96	118,825.77	
Prepaid Insurance		6,605.40	
Items in Suspense (Debits)		686.80	256.750.01
			$2,764,928.70

Liabilities

Endowment Funds:			
Endowment Funds	967,974.68		
Reserve for Amortization	2,443.95	970,418.63	
Minor Funds		21,198.88	991,617.51
Plant Funds:			
Mortgage Note Payable		5,000.00	
Donations and Gifts	1,404,441.81		
Other Investments in Plant from Gifts and Current Funds	107,119.37	1,511,561.18	1,516,561.18
Current Liabilities and Surplus:			
Accounts Payable		31,473.84	
Items in Suspense (Credits)		3,549.31	
Current Surplus		221,726.86	256.750.01
			$2,764,928.70

Marine Biological Laboratory

Five Year Condensed Income and Expense Statement

Reference—Exhibit B Auditors' Reports—1948 through 1952

	1952	1951	1950	1949	1948	Total
Income from Endowments	$ 44,115.00	$ 44,898.43	$ 46,431.59	$ 40,347.15	$ 37,586.48	$ 213,378.65
Income from Investments, Including Retirement Fund and Mortgage Rec.	21,635.50	23,184.86	22,844.19	22,593.55	25,570.93	115,829.03
Income from Fees and Dues, including Instruction, Research, Library	46,695.76	46,633.00	47,888.61	44,921.33	40,321.04	226,459.74
Income from Mess, Dormitories, Supply, Biol. Bulletin, Sundry—Net	19,690.73	12,943.63	21,601.35	27,499.18	28,387.61	110,122.50
Income from Rents, including Bar Neck	7,333.26	8,047.42	7,090.89	6,330.00	6,360.00	35,161.57
TOTAL ORDINARY INCOME A	139,470.25	135,707.34	145,856.63	141,691.21	138,226.06	700,951.49
Income by Special Donations	65,242.69	77,730.00	43,130.66	37,459.45	29,375.00	252,937.80
TOTAL INCOME X	$204,712.94	$213,437.34	$188,987.29	$179,150.66	$167,601.06	$ 953,889.29
Buildings, Grounds, and Other Property Expense	$ 51,020.70	$ 56,353.84	$ 53,066.36	$ 41,591.16	$ 39,536.68	$ 241,568.74
Research Service Expense—Apparatus and Chemical Rooms	24,312.98	25,492.99	25,244.54	23,901.70	24,721.91	123,674.12
Instruction, Research and Library Expense	34,632.76	34,083.50	34,077.76	30,587.06	30,217.79	163,598.87
General Expenses, Administration, Pension Fund, Bank Charges, etc.	39,396.93	41,375.04	37,623.08	43,650.91	38,039.39	200,085.35
	149,363.37	157,305.37	150,011.74	139,730.83	132,515.77	728,927.08
Depreciation	34,359.93	32,181.00	29,160.29	27,919.94	26,666.86	150,288.02
	183,723.30	189,486.37	179,172.03	167,650.77	159,182.63	879.215.10
Plant Additions from Current Funds	19,360.99	16,767.59	17,775.06	33,408.40	49,321.22	136,633.26
TOTAL ORDINARY EXPENSE B	203,084.29	206,253.96	196,947.09	201,059.17	208,503.85	$1,015,848.36
Special Expenditures from Donations	10,433.11	9,705.88	5,129.49	......	.....	25,268.48
	213,517.40	215,959.84	202,076.58	201,059.17	208,503.85	1,041,116.84
Plant Additions from Donations	23,722.13	44,020.64	.....	.....	.....	67,742.77
TOTAL EXPENSE Y	$237,239.53	$259,980.48	$202,076.58	$201,059.17	$208,503.85	$1,108,859.61
TOTAL NET (X – Y)	**32,526.59**	**46,543.14**	**13,089.29**	**21,908.51**	**40,902.79**	**154,970.32**
Delete Depreciation	34,359.93	32,181.00	29,160.29	27,919.94	26,666.86	150,288.02
Total Net, less Depreciation	1,833.34	**14,362.14**	16,071.00	6,011.43	**14,235.93**	**4,682.30**
Operating Net (A – B)	**63,614.04**	**70,546.62**	**51,090.46**	**59,367.96**	**70,277.79**	**314,896.87**
Delete Depreciation	34,359.93	32,181.00	29,160.29	27,919.94	26,666.86	150,288.02
Operating Net, Less Depreciation	**$ 29,254.11**	**$ 38,365.62**	**$ 21,930.17**	**$ 31,448.02**	**$ 43,610.93**	**$ 164,608.85**

Marine Biological Laboratory Current Surplus December 31, 1952

Balance, January 1, 1952			$217,152.46
Add:			
Excess of Income over Expense for Year		$10,556.53	
Prior year's adjustments, Net Recovery		2,741.06	
Reserve for Depreciation, 1952, Charged to Plant Funds		34,359.93	47,657.52
			264,809.98
Deduct:			
Payments during year for Plant Assets:			
Equipment:			
From Current Funds	$ 322.50		
From Rockefeller Foundation Grant for Special Apparatus	20,711.19		
From Lillie Memorial Fund	3,010.94	24,044.63	
Library		13,038.49	
		37,083.12	
Mortgage Note Payment, Elliot House		6,000.00	43,083.12
Balance, December 31, 1952			$221,726.86

Summary of Bank Balance

Cash Balance January 1, 1952		
The Hanover Bank	$ 2,923.71	
Falmouth National Bank	930.25	$ 3,853.96
Receipts		373,097.64
		376,951.60
Payments		348,856.48
Cash Balance, December 31, 1952		
The Hanover Bank	25,547.59	
Falmouth National Bank	2,547.53	28,095.12

Other Cash Accounts	Balances Jan. 1, 1952	Received	Paid	Balances Dec. 31, 1952
Allen R. Memhard Fund	$ 104.15	$ 27.50	$ 45.00	$ 86.65
Rev. Arsenious Boyer Fund	298.82	148.76	270.00	177.58
Lucretia Crocker Fund	739.83	452.55		1,192.38
G. H. A. Clowes Beach Fund	1,000.00			1,000.00
F. R. Lillie Memorial Research Fund	7,986.81	6,225.00	7,818.74	6,393.07
Retirement Fund	2,495.45			
Bio Club Scholarship Fund	127.24	873.76	206.25	794.75
Lalor Foundation Fellowship Fund	1,354.42	6,000.00	6,377.92	976.50

Respectfully submitted,
JAMES H. WICKERSHAM,
Treasurer

V. REPORT OF THE LIBRARIAN

1952

The appropriation for 1952 Library expenses was $15,128 plus $4500 (to be applied to staff salaries) from the Woods Hole Oceanographic Institution. This sum was expended as follows: Serials, $5650.57; Books, $613.79; Back Sets, $74.51; Binding, $2351.90; Supplies, Sundries, Freight and Express, $411.49; Insurance, $55.00; and Salaries, $8328.25; making a total of $17,485.51.

The yearly allowance from the Woods Hole Oceanographic Institution, to acquire oceanographic books and journals and the binding of same, was $1200 plus a balance of $132.08 from 1951. Of this amount, $977.04 was expended, leaving a balance of $355.04 on December 31, 1952, which will be used for an outstanding back-set order.

The titles of current journals received in 1952 numbered 1479 (65 new). There were 450 (6 new) Marine Biological Laboratory subscriptions; 596 exchanges (24 new) and 164 gifts (12 new); 72 (4 new) were Woods Hole Oceanographic Institution subscriptions; 168 (13 new) were exchanges and 29 (6 new) were gifts.

The Marine Biological Laboratory purchased 55 books, received 82 complimentary copies (7 from authors and 75 from publishers) and 16 miscellaneous donations. The Woods Hole Oceanographic Institution purchased 29 titles and received 18 gifts. The total number of new books placed on the shelves was 200.

During World War II, there occurred hiatuses in the current receipts of foreign periodicals. In 1952, four complete volumes and 50 odd numbers were secured, greatly lowering the number of missing items. In a few years, it is hoped that all gaps will be filled in, either in the original or in photographic copy. The Woods Hole Oceanographic Institution partially completed four back sets by purchase and one complete set and one partially complete set were received as gifts.

The reprint collection was increased by 4146 papers, 2032 being of current issue and the remaining ones of earlier dates.

Ninety-three titles were borrowed on inter-library loan and 111 were sent to out-of-town institutions. There were 46 microfilm orders filled, the service earning $273.91. (It was necessary to suspend the service the latter part of the year due to a change in the staff.) A sum of $211.28 was realized from the sale of duplicate material.

The second edition of the list of journal holdings was published in the February issue of the BIOLOGICAL BULLETIN. There are 2680 titles with holdings listed, 1432 of these being of current issue.

In 1952, the Library had the great fortune of receiving the remainder of Dr. Rudolph Höber's collection of reprints on physiological chemistry. The mass of his collection was presented to the Laboratory in 1942. This valuable gift totalled 12,000 papers. In appreciation, a bronze plaque with appropriate inscription was placed in the Library.

At the end of the year 1952, the Library contained 62,151 bound volumes and 182,941 reprints.

For the helpfulness and advice so willingly contributed by the members of the Library Committee, the Librarian wishes to express grateful thanks and appreciation.

Respectfully submitted,
DEBORAH L. HARLOW,
Librarian

VI. REPORT OF THE DIRECTOR

TO THE TRUSTEES OF THE MARINE BIOLOGICAL LABORATORY:

Gentlemen:

I submit herewith a report of the sixty-fifth session of the Marine Biological Laboratory.

The Laboratory continues its progress in the development of its various services. There was added to our equipment this year an electron microscope purchased from the equipment grant of $75,000 made to the Laboratory by the Rockefeller Foundation. Use of the instrument was supervised by Dr. James Hillier. It was in almost continuous daily operation. The total number of scientific services now rendered by the Service Department number 9 which, in addition to the electron microscope, include the radioisotopes laboratory service, X-ray service, special apparatus service (ultracentrifuge, preparation centrifuge, refrigerated centrifuge, freezing-drying apparatus, electrophoresis apparatus, etc.), apparatus supply and repair service, chemistry supply service, glassblowing, carpenter shop service, and biological collecting and supply service.

1. *Changes in Officers:*

This year was marked by the retirement of Mr. Lawrason Riggs as President of the Corporation. Mr. Riggs served the Laboratory faithfully and ably as Treasurer from 1924 to 1942. In 1942 he succeeded Dr. Frank R. Lillie as President of the Corporation. Mr. Riggs served during the trying war years when the problems of the Laboratory were most difficult. The period since then has been marked by a physical rehabilitation of the Laboratory, many plant improvements including a complete rebuilding of Old Main, marked expansion of our research equipment and services and improvement of our biological collecting facilities. The Laboratory was fortunate in having such able leadership during this critical period.

Mr. Riggs was succeeded by Mr. Gerard Swope, Jr., Vice-President of the General Electric Company, who brings to the position deep interest and great enthusiasm.

Dr. E. Newton Harvey retired at the end of the year as Vice-President of the Corporation. It was with great reluctance that the Trustees acceded to Dr. Harvey's wish to retire. His broad scientific knowledge and excellent judgment were invaluable during his 11 year term as Vice President.

Dr. Arthur K. Parpart, who has been in regular attendance at the Laboratory since 1924 and who is so well-versed in its problems, was elected to succeed Dr. Harvey.

Mr. Donald M. Brodie who, since 1942, has served the Laboratory as Treasurer of the Corporation, also requested retirement. The Laboratory is grateful for his years of service and his wise counsel in our financial affairs.

Mr. James H. Wickersham, Vice-President of the Bank of New York, succeeded Mr. Brodie and here again the Laboratory was fortunate in acquiring the support and assistance of a man with broad experience to advise us.

2. *Instruction:*

The course in Marine Ecology in its initial year operated most effectively. A large number of students and investigators from the three Woods Hole Institutions monitored the course in which there was a sustained interest. The staff under the direction of Dr. Bostwick H. Ketchum is to be commended on the results they achieved. The New York Zoological Society has renewed its grant of $4,000 for another year's support of this course.

3. *Increases in Rates:*

When the Executive Committee met at the end of the year to set up the budget for 1953, it was immediately obvious that there would be a sizable deficit unless new funds were obtained. The salaries paid the full time staff had lagged considerably behind the increased costs of living since the war so that increases were essential. A part of this will be met by an average 20% increase in the laboratory rental space. Current negotiations with the National Science Foundation and the Office of Naval Research may provide additional funds through contracts which will help cover the prospective deficit.

4. *The Bar Neck Property:*

In November the Government instituted condemnation proceedings for the taking of the Bar Neck Property as the site for its new Oceanographic Laboratory. The M. B. L. could fight condemnation; however, this would be very expensive and fruitless according to legal advice obtained by the Executive Committee. During the war the Armed Services were given unusual powers of condemnation which have not yet been rescinded. The loss of this property is a serious blow to the Laboratory, both income-wise and in terms of its future development. The Bar Neck site appears to have been chiefly a matter of convenience rather than one of necessity since there were other locations on which the Government could have put its new laboratory. Also, it appears that The Marine Biological Laboratory will be unable to recover adequate damages for the loss of the property since some of the features which made it so valuable to the Laboratory probably will not be considered pertinent in an appraisal.

5. *Grants, Contributions and Gifts:*

These are assuming great importance in the support of the Laboratory. This past year they totaled $65,242.69.

Grants:	
Rockefeller Foundation, general support	$17,500
Rockefeller Foundation, special apparatus (balance)	25,000
American Cancer Society, radioisotopes laboratory	1,000
Kettering Foundation, general support	2,500
American Philosophical Society, laboratory support	1,800
New York Zoological Society, ecology course	4,000
Contributions:	
Eli Lilly Company	5,000
Mr. Lawrason Riggs	1,000
Dr. Charles Kettering	2,500
Mrs. W. Murray Crane	1,300
Dr. W. D. Curtis	463

Gifts:

Mr. H. D. Loring has presented to the Laboratory a new 25-foot power boat which will be an important addition to the fleet of boats used by the Supply Department for the collection of biological materials. The name chosen for the boat, "Loligo," is in the Laboratory tradition, the name of a marine form used extensively in research.

The generosity of the various supporters of the Laboratory is deeply appreciated by the Corporation of the Marine Biological Laboratory. It is also a most gratifying expression of their faith in the Laboratory.

6. *M. B. L. Associates:*

The Associates of the Laboratory now number 95. They contributed $1610 to the Laboratory this past year, which has been ear-marked for the reconstruction of the marine railway. A part of this work will be carried out before the summer of 1953.

7. *Deaths:*

The Corporation suffered the loss this year of one of its most distinguished and oldest members with the death of Dr. Edwin Grant Conklin who played such an important role in the development of the Laboratory during its formative years. His researches were also among the most notable coming from the Laboratory.

Respectfully submitted,
Philip B. Armstrong,
Director

1. Memorial

Ralph Stayner Lillie

1875–1952

By

Dr. E. G. Conklin

In the death of Ralph Lillie on March 19, 1952, the Marine Biological Laboratory lost one of its early and most constant members and the world of science and philosophy a scholar of distinction. He was born in Toronto, Canada, August 8, 1875, about five years after the birth of his brother, Frank R. Lillie, and his education, culminating in the degree A.B. from the University of Toronto in 1896 and of Ph.D. from the University of Chicago in 1901, followed by five years the same degrees from the same universities conferred upon his brother Frank. In spite of these similarities in their origin and education Ralph Lillie was from the first a scientist and scholar of marked individuality; his chosen field of work was general physiology and its relations to the great problems of psychology and philosophy, rather than the prevailing work in morphology and morphogenesis, which characterized so much of the research of this Laboratory in the early years of this century.

After receiving the doctor's degree at the University of Chicago in 1901, he was an assistant in physiology at Harvard for one year, and then instructor and adjunct professor of physiology at the University of Nebraska for three years; in 1904–05 he was appointed by the Carnegie Institution of Washington, as one of its research assistants to occupy a table in the Naples Zoological Station, and this was followed by one year as instructor in physiology at Harvard and by another as Johnston Scholar at Johns Hopkins University.

At that time physiology was generally regarded as a strictly medical school subject, with emphasis on the physiology of higher animals and men, and with small place for such general physiology as Ralph Lillie was committed to, but in 1907 he was appointed, under my selection, assistant professor of general physiology and experimental zoology in the Department of Zoology at the University of Pennsylvania. He continued in that position until 1913 when he was called to be professor of biology in Clark University. In 1920 he was appointed research biologist in the laboratory of the National Electric Light Association in Cleveland, and after four years in that position he finally came in 1924 to the professorship of general physiology in the University of Chicago, which position he held with honor and distinction until his retirement for age in 1940, when he became professor emeritus.

Dr. Lillie was a thinker and writer rather than a public speaker and while he was a member of most of the national societies of biology, physiology, and philosophy, including the two oldest and most distinguished learned societies of America, he rarely attended their meetings or took part in their programs, owing in part to a slight defect in hearing. One must, therefore, depend largely on his publications in estimating the character and extent of his work. In the library of the Marine Biological Laboratory there are 120 reprints of his research papers and two books, one of these in two editions, which are listed in the card catalogue under some 20 topics in general physiology, while his philosophical writings are grouped together under the topic "theoretical biology." About 96 of these reprints are primarily in general biology, while 24 are more largely philosophical. It is significant that he published four times as many papers in physiology as in philosophy and that all of his papers from 1901 to 1914 were in physiology, while almost all in the last decade of his life were philosophical. In short, his philosophy was an outgrowth of his physiology and an attempt to fit the facts of biology—all facts of life including human life—into a logical and practical system.

Among his more important contributions to physiology should be mentioned the effects of various salt solutions on ciliary and muscular movement; relations of ions to contractility and irritability; influence of electrolytes on osmotic pressure of colloidal solutions; the connection between permeability of plasma membranes and irritability, contractility and living processes in general; and, growing out of this, some seven papers on the physiology of anaesthesia. Four main papers were devoted to the physiology of cell division in which he pointed out similarities between certain floating bodies in electrical fields and the positions of chromosomes in mitosis—a model which has been widely used in teaching cytology. Several papers were on the rate of conduction in nerve and irritable tissues, and in connection with this he invented the iron-wire model of such transmission, recovery and rhythmical activity, which is now widely used in explaining these phenomena. This is merely a brief mention of some of his more important and original contributions to general physiology.

In philosophy he was not content to leave out of account all phenomena not now explicable by current materialistic theories, or to explain them by explaining them away as non-existent. He maintained that man and all his psychical qualities must be included in any comprehensive philosophy of nature, for he is a part of nature. He defined his outlook on such a philosophy as one of "critical naturalism" and he held that present mechanistic science is based too largely on physics and static conditions, and takes too little account of biology, creative synthesis and novelty. He held that "every living organism is the psycho-physical unity," not a duality of soul and body, and in general he agreed with the philosophy of Alfred North Whitehead. It is difficult and indeed impossible in a brief summary, to do justice to his profound philosophy but it is summarized in his last book, General Biology and Philosophy of Organism (University of Chicago Press, 1945).

Throughout his whole professional life Ralph Lillie was intimately associated with the Marine Biological Laboratory. In the summer of 1896, immediately following his graduation from the University of Toronto, he was a graduate student in the embryology class here, and according to the Annual Reports of the Laboratory, and the memories of friends and associates, he was present as investigator or instructor for at least a part of every summer since 1896, a total of 55 sessions, which is almost a unique record. From 1902 to 1922 he was instructor in the class in comparative physiology, and thereafter until 1941 he was a member of the "staff of instruction in physiology," since which time he has been recorded as a member of the "senior staff of instruction." In 1921 he was elected a Trustee of the Laboratory for a term of four years and on five different occasions since then at the expiration of his term he was re-elected to the board; finally in 1945 he was elected trustee emeritus.

Not only did Ralph Lillie's research life have its center here at Woods Hole but some of his most intimate social and family relations also centered here. It is a notable fact that many, perhaps a majority of the earlier workers at the Laboratory, who were not already married, met their matrimonial mates here among students and co-workers and such marriages have been both life-long and eugenic; it is a record of which this Laboratory may well be proud. Here Ralph Lillie and Helen Makepeace met, became engaged, and in 1906 were married, and here they have ever since made their summer home, which has been blessed by two worthy sons, and which in turn has blessed many friends who have enjoyed their charming hospitality. Dr. Lillie was a pianist of note and the artistic and literary quality of their home added much to the cultural atmosphere of Woods Hole.

It is not surprising that those who have lived and worked here and have enjoyed the associations and pleasures of this place should love it beyond all others, nor that they should cherish the hope of leaving not only their thoughts and examples but also their mortal remains in this Valhalla of Biologists. This was the desire of Ralph Lillie and his family, and after a beautiful funeral service in the Church of the Messiah on April 3, 1952, his ashes were buried in the churchyard here where rest so many of our honored dead.

2. The Staff, 1951

Philip B. Armstrong, Director, State University of New York, School of Medicine, Syracuse.

Senior Staff of Investigation

E. G. Conklin, Professor of Zoology, *Emeritus,* Princeton University.
A. P. Mathews, Professor of Biochemistry, *Emeritus,* University of Cincinnati.
G. H. Parker, Professor of Zoology, *Emeritus,* Harvard University.

ZOOLOGY

I. Consultants

F. A. Brown, Jr., Professor of Zoology, Northwestern University.
Libbie H. Hyman, American Museum of Natural History.
A. C. Redfield, Woods Hole Oceanographic Institution.

II. Instructors

L. H. Kleinholz, Associate Professor of Biology, Reed College, in charge of course.
George M. Moore, Professor of Zoology, University of New Hampshire.
C. G. Goodchild, Professor of Biology, S. W. Missouri State College.
John H. Lochhead, Associate Professor of Zoology, University of Vermont.
Madelene E. Pierce, Associate Professor of Zoology, Vassar College.
Marion H. Pettibone, Arctic Research Laboratory, U. S. National Museum.
Nathan W. Riser, Department of Biology, Fisk University.
Joan C. Rattenbury, Department of Zoology, McGill University.

III. Laboratory Assistants

Robert S. Howard, Northwestern University.
Elizabeth Paulsen, Rutgers University.

EMBRYOLOGY

I. Instructors

S. Meryl Rose, Associate Professor of Zoology, University of Illinois, in charge of course.
Charles B. Metz, Assistant Professor of Zoology, Yale University.
John T. Bonner, Assistant Professor of Biology, Princeton University.
Mac V. Edds, Jr., Associate Professor of Biology, Brown University.
John R. Shaver, Assistant Professor of Zoology, University of Missouri.
Edgar Zwilling, Associate Professor of Genetics, University of Connecticut.

II. Laboratory Assistants

Lillian M. Youngs, University of North Carolina.
Joseph G. Gall, Yale University.

PHYSIOLOGY

I. Consultants

Merkel H. Jacobs, Professor of Physiology, University of Pennsylvania.
Otto Loewi, Professor of Pharmacology, New York University, School of Medicine.
Arthur K. Parpart, Professor of Biology, Princeton University.
Albert Szent-Gyorgyi, Director, Institute for Muscle Research.

II. Instructors

Daniel Mazia, Associate Professor of Zoology, University of California.
Michael Doudoroff, Associate Professor of Bacteriology, University of California.
Irving M. Klotz, Professor of Chemistry, Northwestern University.
Stephen Kuffler, Associate Professor, Johns Hopkins University.
H. Burr Steinbach, Professor of Zoology, University of Minnesota.
George Wald, Professor of Biology, Harvard University.
Milton Levy, Associate Professor of Chemistry, New York University College of Medicine.
Robert C. Warner, Assistant Professor of Chemistry, New York University College of Medicine.

III. Laboratory Assistant

Paul Bernstein, Washington University.

BOTANY

I. Consultants

Maxwell S. Doty, Associate Professor of Botany, University of Hawaii.
Wm. Randolph Taylor, Professor of Botany, University of Michigan.

II. Instructors

Francis T. Haxo, Assistant Professor of Biology, Johns Hopkins University, in charge of course.
Paul C. Silva, Department of Botany, University of California.
Richard C. Starr, Instructor in Botany, University of Indiana.

III. Laboratory Assistant

Phyllis M. Strout, Yale University.

IV. Collector

Peter Jennings, Drew University.

V. Lecturers

J. B. Lackey, Medical and Scientific Editor, The Blakiston Company.
John Wallace, Academy of Natural Sciences of Philadelphia.

MARINE BIOLOGICAL

MARINE ECOLOGY

I. *Consultants*

W. C. Allee, University of Florida.
Alfred C. Redfield, Woods Hole Oceanographic Institution.

II. *Instructors*

Bostwick H. Ketchum, Marine Microbiologist, Woods Hole Oceanographic Institution, in charge of course.
Edwin T. Moul, Assistant Professor of Botany, Rutgers University.
Charles Jenner, Assistant Professor of Zoology, University of North Carolina.

EXPERIMENTAL RADIOLOGY

G. Failla, College of Physicians and Surgeons, Columbia University.
L. Robinson Hyde, Phillips Exeter Academy, Exeter, N. H.
Mones Berman, Memorial Cancer Center, New York City.

LIBRARY

Mrs. Deborah Lawrence Harlow, Librarian
Mary A. Rohan

APPARATUS DEPARTMENT

Robert Mills, Manager
James D. Graham
Betty J. Keeler
Seaver R. Harlow

CHEMICAL DEPARTMENT

Gail M. Cavanaugh, Chemical Supplies Manager

SUPPLY DEPARTMENT

James McInnis, Manager
John S. Rankin, Naturalist
Ruth Crowell
Patricia M. Conway

M. B. Gray
A. M. Hilton
W. E. Kahler

G. Lehy
Carl O. Schweidenback
R. O. Lehy

James Whitcomb
H. S. Wagstaff
Robert Perry

GENERAL OFFICE

Homer P. Smith, General Manager
Polly L. Crowell, Office Manager
Mrs. Lila S. Myers
Nancy Shave

GENERAL MAINTENANCE

R. W. Kahler, Superintendent

Robert Adams
Richard Alberts
Robert Gunning
John H. Head

James S. Thayer
Albert Neal
Alton J. Pierce
T. E. Tawell

George A. Kahler

3. INVESTIGATORS AND STUDENTS

Independent Investigators, 1952

ALLEE, W. C., Professor of Biology, University of Florida.
ALLEN, M. JEAN, Assistant Professor of Zoology, University of New Hampshire.
ALSCHER, RUTH P., Assistant Professor of Biology, Manhattanville College of the Sacred Heart.
ANDERSON, RUBERT S., Professor of Physiology, University of South Dakota.
ARMSTRONG, PHILIP B., Professor of Anatomy, State University of New York at Syracuse.
BALL, ERIC G., Professor of Biological Chemistry, Harvard University Medical School.
BANG, FREDERICK B., Associate Professor of Medicine, Johns Hopkins Hospital.
BARTLETT, JAMES H., Professor of Physics, University of Illinois.
BARTON, JAY, II, Instructor in Zoology, Columbia University.
BERGER, CHARLES A., Director, Biological Laboratory, Fordham University.
BLUM, HAROLD F., Physiologist, NCI, and Visiting Lecturer, Princeton University.
BOETTIGER, EDWARD G., Assistant Professor of Zoology, University of Connecticut.
BONNER, JOHN T., Associate Professor of Biology, Princeton University.
BOYCE, STEVE G., Teaching Fellow, North Carolina State College.
BOYER, PAUL D., Associate Professor Ag. Biochemistry, University of Minnesota.
BRONK, DETLEV, President, Johns Hopkins University.
BROWN, FRANK A., JR., Professor and Chairman Biological Sciences, Northwestern University.
BUTLER, E. G., Professor of Zoology, Princeton University.
CHASE, AURIN M., Associate Professor of Biology, Princeton University.
CHENEY, RALPH H., Professor of Biology, Brooklyn College.
CIERESZKO, LEON S., Assistant Professor of Chemistry, University of Oklahoma.
CLAFF, C. LLOYD, Research Associate in Surgery, Harvard Medical School.
CLARK, ARNOLD M., Associate Professor of Biology, University of Delaware.
CLARK, ELIOT R., Professor of Anatomy, *Emeritus,* University of Pennsylvania School of Medicine.
CLEMENT, A. C., Associate Professor of Biology, Emory University.
CLOWES, G. H. A., Research Director, *Emeritus,* Eli Lilly and Company.
COHEN, SEYMOUR S., Associate Professor, Childrens Hospital, University of Pennsylvania.
COLE, KENNETH S., Technical Director, Naval Medical Research Institute.
COLWIN, ARTHUR L., Associate Professor of Biology, Queens College.
CONNELLY, CLARENCE M., Assistant Professor of Biophysics, Johns Hopkins University.
COOPERSTEIN, SHERWIN J., Senior Instructor in Anatomy, Western Reserve University.
COPLEY, ALFRED L., Assistant Clinical Professor, New York Medical College.
CORNMAN, IVOR, Assistant Research Professor in Anatomy, George Washington University.
COSTELLO, DONALD P., Kenan Professor of Zoology, University of North Carolina.
CRANEFIELD, PAUL F., Post-doctorate Fellow, Johns Hopkins University.
CROWELL, SEARS, Assistant Professor of Zoology, Indiana University.
CSAPO, ARPAD I., Staff Member and Lecturer, Carnegie Institution of Washington.
CURTIS, W. C., Professor of Zoology, *Emeritus,* University of Missouri.
DISTEFANO, HENRY S., Instructor in Anatomy, State University of New York at Syracuse.
DOUDOROFF, MICHAEL, Associate Professor of Bacteriology, University of California.
DUBOIS, EUGENE F., *Emeritus* Professor of Physiology, Cornell University Medical College.
EDDS, M. V., JR., Associate Professor of Biology, Brown University.
EICHEL, BERTRAM, Assistant Research Specialist, Rutgers University.
EICHEL, HERBERT J., Research Associate, Hahnemann Medical College.
ELLIOTT, ALFRED M., Associate Professor of Zoology, University of Michigan.
FAILLA, G., Professor of Radiology, Columbia University.
FIGGE, FRANK H. J., Professor of Anatomy, University of Maryland Medical School.
FLORKIN, MARCEL, Professor, University of Liège, Belgium.
FRAENKEL, GOTTFRIED, Professor of Entomology, University of Illinois.
FREEMAN, PAUL J., Instructor in Physiology, San Jose State College.
FREYGANG, WALTER, JR., National Institute of Health.
GASTEIGER, EDGAR L., Research Fellow, Massachusetts General Hospital.

Gilman, Lauren C., Associate Professor of Zoology, University of Miami.
Goodchild, Chauncey G., Professor of Biology, S. W. Missouri State College.
Goodrich, Hubert B., Professor of Biology, Wesleyan University.
Grant, Philip, Public Health Fellow, Columbia University.
Griffin, Donald R., Associate Professor of Zoology, Cornell University.
Grosch, Daniel S., Associate Professor, Biological Division, North Carolina State College.
Grundfest, Harry, Associate Professor of Neurology, College of Physicians and Surgeons.
Gudernatsch, Frederick, 41 Fifth Avenue, New York 3, New York.
Guttman, Rita, Assistant Professor, Brooklyn College.
Harvey, Ethel Browne, Independent Research Investigator, Princeton University.
Harvey, E. Newton, Professor of Physiology, Princeton University.
Haxo, Francis, Assistant Professor of Biology, Johns Hopkins University.
Hayashi, Teru, Assistant Professor of Zoology, Columbia University.
Haywood, Charlotte, Professor of Physiology, Mount Holyoke College.
Heilbrunn, L. V., Professor of Zoology, University of Pennsylvania.
Hendley, Charles D., Assistant Professor of Experimental Neurology, Tulane University.
Henley, Catherine, Research Associate, University of North Carolina.
Hibbard, Hope, Professor of Zoology, Oberlin College.
Inoué, Shinya, Instructor in Anatomy, University of Washington, School of Medicine.
Jacob, Miriam I., Teaching Assistant, New York University.
Jacobs, Merkel H., Professor of General Physiology, University of Pennsylvania.
Jenkins, George B., Professor of Anatomy, *Emeritus,* George Washington University.
Jenner, Charles E., Assistant Professor of Zoology, University of North Carolina.
Kao, Chien-juan, Assistant in Pathology, Cornell University Medical School.
Kavanau, J. Lee, Research Fellow, University of California.
Kempton, Rudolf T., Professor of Zoology, Vassar College.
Keosian, John, Professor of Biology, Rutgers University.
Kind, Charles Albert, Assistant Professor of Chemistry, University of Connecticut.
King, John W., Professor of Biology, Morgan State College.
Kleinholz, L. H., Professor of Biology, Reed College.
Krahl, Maurice E., Associate Professor Biological Chemistry, Washington University School of Medicine.
Kuffler, Stephen W., Associate Professor, Johns Hopkins University.
Kuntz, Eloise, Instructor in Physiology, Vassar College.
Lansing, Albert I., Associate Professor of Anatomy, Washington University School of Medicine.
Lazarow, Arnold, Associate Professor, Western Reserve University.
Levy, Milton, Associate Professor, New York University College of Medicine.
Ling, Gilbert, Instructor in Physiological Optics, Wilmer Institute, Johns Hopkins Hospital.
Lochhead, John H., Professor of Zoology, University of Vermont.
Love, Warner E., Research Fellow, University of Pennsylvania.
Lynch, William F., Professor of Biology, St. Ambrose College.
Makino, Sajiro, Professor of Zoology, Hokkaido University, Sapporo, Japan.
Marsland, Douglas, Professor of Biology, Washington Square College, New York University.
Mankowski, Zbigniew T., Research Fellow, Cancer Institute.
Marshak, Alfred, 32 Orange Street, Brooklyn 2, New York.
Mazia, Daniel, Associate Professor of Zoology, University of California.
Menkin, Valy, Associate Professor of Experimental Pathology, Temple University School of Medicine.
Metz, Charles B., Assistant Professor of Zoology, Yale University.
Moore, John W., Biophysicist, Naval Medical Research Institute.
Moul, Edwin T., Assistant Professor of Botany, Rutgers University.
Mullahy, John H., Assistant Professor of Biology, Loyola University.
Musacchia, X. J., Assistant Professor, Biological Labs., St. Louis University.
Nachmansohn, David, Associate Professor of Neurology, Columbia University.
Newton, L. M., A. A. U. W. Fellow, British Museum (Natural History) London.

Nichol, Charles A., Senior Instructor in Pharmacology, Western Reserve School of Medicine.
Nieuwkoop, Pieter D., Research Fellow, Hubrecht Lab., Utrecht, Netherlands.
Osterhout, W. J. V., Member, *Emeritus,* Rockefeller Institute.
Parmenter, Charles L., Professor of Zoology, University of Pennsylvania.
Parpart, Arthur K., Chairman, Department of Biology, Princeton University.
Perkins, John F., Assistant Professor of Physiology, University of Chicago.
Person, Philip, USPHS Post Doctoral Research Fellow, Rutgers University.
Pettibone, Marian H., Research Associate, Johns Hopkins University.
Pierce, Madelene E., Professor of Zoology, Vassar College.
Proctor, Nathaniel K., Associate Professor of Biology, Morgan State College.
Prosser, C. Ladd, Professor of Physiology, University of Illinois.
Rattenbury, Joan C., Lecturer in Zoology, McGill University.
Ray, David T., Instructor of Zoology, Howard University.
Reverberi, Giuseppe, Professor of Zoology, Universita Palermo, Italy, via Archirafi,18.
Riser, Nathan W., Associate Professor of Biology, Fisk University.
Ronkin, R. R., Assistant Professor of Physiology, University of Delaware.
Roofe, Paul G., Chairman and Professor of Anatomy, University of Kansas.
Rose, S. Meryl, Associate Professor of Zoology, University of Illinois.
Roth, Jay S., Assistant Professor of Biochemistry, Hahnemann Medical College and Hospital.
Rugh, Roberts, Associate Professor of Radiology, Columbia University.
Sandeen, Muriel I., Instructor in Zoology, Duke University.
Schaeffer, Asa A., Professor of Biology, Temple University and Biological Institute.
Scharrer, Ernst A., Associate Professor, University of Colorado School of Medicine.
Schechter, Victor, Assistant Professor of Biology, The City College of New York.
Sclufer, Evelyn, Graduate Student, University of Pennsylvania.
Scott, Sister Florence Marie, Professor of Biology, Seton Hill College.
Scott, Allan, Professor of Biology, Colby College.
Scott, Dwight B. McNair, Associate in Biochemistry, Children's Hospital of Philadelphia.
Shanes, A. M., Physiologist, National Institutes of Health.
Shaver, John, Assistant Professor of Zoology, University of Missouri.
Sichel, F., Professor of Physiology and Biophysics, University of Vermont, College of Medicine.
Silva, Paul C., Research Associate in Botany, University of California.
Slifer, Eleanor H., Associate Professor of Zoology, State University of Iowa.
Solomon, Sidney, Instructor in Physiology, Medical College of Virginia.
Sonnenblick, B. P., Associate Professor of Biology, Rutgers University.
Speidel, Carl C., Professor and Chairman, School of Anatomy, University of Virginia.
Spratt, Nelson T., Jr., Professor of Zoology, University of Minnesota.
Stadler, David R., Instructor in Biology, University of Rochester.
Stokey, Alma C., Professor of Plant Science, *Emeritus,* Mount Holyoke College.
Strittmatter, Cornelius F., Harvard Medical School.
Sturtevant, A. H., Professor of Biology, California Institute of Technology.
Taylor, William Randolph, Professor of Botany, University of Michigan.
Tobias, Julian M., Associate Professor of Physiology, University of Chicago.
Tracy, Henry C., Professor of Anatomy, *Emeritus,* University of Kansas.
Trager, William, Associate Member, Rockefeller Institute.
Trinkaus, J. P., Assistant Professor of Zoology, Yale University.
Truant, Aldo P., Assistant Professor of Pharmacology, Tufts Medical School.
Warner, Robert C., Assistant Professor of Chemistry, New York University College of Medicine.
Watterson, Ray L., Associate Professor of Biology, Northwestern University.
Webb, H. Marguerite, Assistant Professor of Biology, Boston College.
Whiting, P. W., Professor of Zoology, University of Pennsylvania.
Wichterman, Ralph, Professor of Biology, Temple University.
Wilber, Charles G., Director, Biological Laboratories, St. Louis University.
Wilson, Walter L., Instructor of Physiology and Biophysics, University of Vermont.
Wright, Paul A., Assistant Professor of Zoology, University of Michigan.

Wrinch, Dorothy, Lecturer in Physics, Smith College.
Zwilling, Edgar, Associate Professor, University of Connecticut.
Yamada, Tuneo, Nagoya University, Japan.

Beginning Investigators, 1952

Allen, Robert Day, Graduate Student, University of Pennsylvania.
Beiser, William, Jr., Student, University of Delaware.
Bernstein, Gerald S., Graduate Student, University of Delaware.
Butros, Joseph, Graduate Student, Emory University.
Chaet, Alfred B., Research Assistant, University of Pennsylvania.
Collier, Jack R., Graduate Student, University of North Carolina.
Conover, John Townsend, Graduate Assistant, University of Michigan.
Conway, John V., Student, University of Delaware.
Couillard, Pierre, Graduate Student, University of Pennsylvania.
Deck, J. David, Graduate Student, Princeton University.
Goldstein, Lester, Graduate Student, University of Pennsylvania.
Graves, Robert C., Graduate Student, Northwestern University.
Greene, Peter Harold, University Fellow in Mathematical Biology, University of Chicago.
Gross, Paul R., Assistant Instructor, University of Pennsylvania.
Hempling, Harold G., Graduate Student, Princeton University.
Herbert, Edward, Graduate Student, University of Pennsylvania.
Herr, Earl B., Jr., Graduate Student, University of Delaware.
Hinton, Claude W., Graduate Student, California Institute of Technology.
Hoffman, Joseph F., Graduate Student, Princeton University.
Honegger, Carol M., Instructor, Temple University.
Horowitz, Iona B., Student, Vassar College.
Ivey, William D., Graduate Student, Emory University.
Katz, Arnold M., University of Chicago.
Kohn, Robert R., Teaching Fellow, University of Michigan.
Landau, Joseph V., Research Assistant, New York University.
McCann, Frances, Graduate Student, University of Connecticut.
McCauley, Molly M., Graduate Student, St. Louis University.
Maroney, Samuel P., Graduate Student, University of Delaware.
Meyer, Marion P., Research Assistant, University of Wisconsin.
Moner, John G., Jr., Graduate Student, Princeton University.
Padykula, Helen A., Research Fellow, Harvard Medical School.
Pandalai, Krishnan R., Assistant Professor, Medical College, Trivandrum, S. India.
Rothman, Walter H., Research Assistant, University of Wisconsin.
Shanklin, Douglas R., Student, New York State College of Medicine at Syracuse.
Slater, John V., Postdoctorate Fellow A.E.C., University of Michigan.
Steinberg, Malcolm S., Graduate Student, Amherst College.
Whiteacre, James F., Instructor in Physiology, Mount Holyoke College.
Wolf, Barbara, Assistant, Rockefeller Institute.

Library Readers, 1952

Andrus, Julian L., Associate Professor of Pharmacology, Philadelphia College of Pharmacology.
Baumberger, J. Percy, Professor of Physiology, Stanford University.
Bodansky, Oscar, Attending Clinical Biochemist, Sloan-Kettering Institute.
Cowgill, Robert W., Instructor in Biochemistry, Washington University School of Medicine.
D'Ancona, Umberto, Professor, Inst. of Zoology, University of Padua, Italy.
Daly, Marie M., Assistant, Rockefeller Institute for Medical Research.
Deane, Helen W., Assistant Professor of Anatomy, Harvard University Medical School.
Dixon, Frank J., Professor of Pathology, University of Pittsburgh, School of Medicine.
Dorfman, Albert, Assistant Professor of Pediatrics, University of Chicago.

Freund, Jules, Chief, Division of Applied Immunology, Public Health Research Institute.
Fries, E. F. B., Assistant Professor, The City College of New York.
Gabriel, Mordecai L., Assistant Professor, Brooklyn College.
Gaffron, Hans, Associate Professor of Biochemistry, University of Chicago.
Ginsberg, Harold S., Associate Professor, Western Reserve University School of Medicine.
Grobstein, Clifford, Biologist, National Cancer Institute.
Gurewich, Vladimir, Assistant Visiting Physician, Bellevue Hospital.
Johnson, Frank H., Associate Professor of Biology, Princeton University.
Hodes, Robert, Professor of Neurophysiology, Tulane University School of Medicine.
Jones, Sarah R., Instructor in Zoology, Connecticut College.
Kabat, Elvin A., Associate Professor of Bacteriology, Columbia University.
Karush, Fred, Assistant Professor of Immunology, Univ. of Pennsylvania School of Medicine
Kiebel, Geraldine, Public Health Service Predoctorate Research Fellow, New York University.
Kramer, Mollie P., Bibliographer, American Meteorological Society.
Kulka, Johannes P., Instructor in Pathology, Harvard Medical School.
Kurnick, Nathaniel B., Assistant Professor of Medicine, Tulane University School of Medicine.
Lindegren, Carl C., Professor, Southern Illinois University.
Loewi, Otto, Research Professor in Pharmacology, New York Univ. Bellevue Medical Center.
Lowenstein, Bertrand E., c/o Dade County Cancer Institute.
Luria, S. E., Professor of Bacteriology, University of Illinois.
McDonald, Sister Elizabeth Seton, Chairman, Dept. of Biology, College of Mt. St. Joseph.
Macey, Robert I., U. S. P. H. S. Research Fellow, University of Chicago.
Nachmansohn, David, Associate Professor, Columbia University.
Namias, Jerome, Chief, Extended Forecast Section, United States Weather Bureau.
Reiner, John M., Assistant Professor of Biochemistry, Columbia University.
Roys, Chester, Research Associate, Tufts College.
Schmidt, Gerhard, Research Professor of Biochemistry, Tufts College Medical School.
Scott, Thomas F. M., Research Professor of Pediatrics, Children's Hospital of Philadelphia.
Selby, Cecily C., Assistant, Virus Study Section, Sloan-Kettering Institute.
Sippel, Theodore O., Graduate Student, Yale University.
Stoloff, Leonard, Research Director, Seaplant Chemical Corporation.
Stunkard, Horace W., Professor of Biology, New York University.
Sulkin, S. Edward, Professor of Bacteriology, Southwestern Medical School.
Tasaki, Ichiji, Research Associate, Central Institute for the Deaf, St. Louis.
Trotter, Mildred, Professor of Gross Anatomy, Washington University.
Uheles, R., Student, New York University.
Wainio, Walter W., Associate Professor of Biochemistry, Rutgers University.
Wetmore, Ralph, Department of Botany, Harvard University.
Zorzoli, Anita, Assistant Professor of Physiology, Southern Illinois University.

Research Assistants, 1952

Altamirano, Mario, Columbia University.
Bergman, Ronald, University of Illinois.
Borysko, Emil, Johns Hopkins University.
Bullowa, Anne, New York Medical College.
Clark, Marguerite R., Western Reserve University.
Cohen, Max, Columbia University.
Cook, John S., Princeton University.
Drake, John W., Yale University.
Engels, Audrey, University of Minnesota.
Ewall, Laroy H., Fisk University.
Fass, Jerome, Rockefeller Institute.
Fingerman, Milton, Northwestern University.
Fox, Howard A., Union College.

Franklin, Malcolm, University of Mississippi.
Frick, Willy, Institute for Muscle Research.
Gall, Joseph G., Yale University.
Glade, Richard W., University of Illinois.
Groesbeck, Marjorie, University of Pennsylvania.
Hayward, Hugh R., Oberlin College.
Horvath, Beni, Carnegie Institution.
Howard, Robert S., Northwestern University.
Jacobs, Jay A. H., Johns Hopkins University.
Joly, Aylthon B., University of Michigan.
Kao, Chun-Juan, Vassar College.
Kaplan, Ann E., Columbia University.
Kaye, Alvin M., Columbia University.
Loos, Gordon M., Harvard University.
Martin, B. Jean, University of Minnesota.
Melton, C. E., University of Illinois.
Rosenblüth, Michael, New York University Medical School.
Rosenbluth, Raja, Columbia University.
Roth, Lorraine B., Children's Hospital of Philadelphia.
Sabal, James, University of Michigan.
Shirer, Hampton W., Naval Medical Research Institute.
Staub, Norman C., State University School of Medicine at Syracuse.
Stearns, Richard N., University of Illinois.
Sullivan, Robert L., North Carolina State College.
Vaughan-Williams, E. M., Wilmer Inst., Johns Hopkins Hospital.
Yarus, Stanley S., Hahnemann Medical College.
Youngs, Lillian M., University of North Carolina.

Students, 1952

BOTANY

Allen, Marjory A., Smith College.
Anderson, Sonya, University of Massachusetts.
Arndt, William F., Jr., Wesleyan University.
Atkin, Adam, Antioch College.
Cormier, Joan, Elmira College.
Dubé, Jean F., Yale University.
Hawthorne, Mary E., Rhode Island University.
Krotkov, N. Sonia, Queen's University.
Marko, Anita R., Adelphi College.
Romig, Robert W., Drew University.
Stein, Janet R., Wellesley College.

ECOLOGY

Bernatowicz, Albert J., National Science Foundation.
Fahey, Elizabeth M., State Teachers College.
Gould, David, Bard College.
Hall, John E., University of New Hampshire.
Howson, Mary Christine, Vassar College.
Polnik, Amelia, University of Chicago.
Scheltema, Rudolf S., George Washington University.

EMBRYOLOGY

Abrams, Leonard S., Columbia University.
Amberson, Barbara D., Jefferson Medical College.
Becker, Simon C., St. Norbert College.

Child, Frank M., III, Amherst College.
Chin, Edward, University of New Hampshire.
Cohen, Mollie, Brooklyn College.
Doak, Anne B., Randolph-Macon Woman's College.
Fimian, Walter J., Jr., University of Notre Dame.
Gautereaux, Sister Miriam Eugene, Marquette University.
Goldman, Allen Seymour, Brown University.
Hey, Robert Vande, St. Norbert College.
Hill, Robert B., Tufts College.
Hillman, Ralph, Yale University.
Ito, Susumu, Western Reserve University.
King, Elizabeth N., Wellesley College.
Krivanek, Jerome O., University of Florida.
Lowry, Joan L., Wellesley College.
McKenzie, John W., University of North Carolina.
Morris, Charles C., Columbia University.
Rafferty, Keen A., Jr., University of Illinois.
Saxer, Robert J., Spring Hill College.
VanGelder, Richard G., University of Illinois.
Warren, Martha M., Mount Holyoke College.

PHYSIOLOGY

Bacila, Metry, Instituto de Biologia, Rua Angelo Sampaio, Brasil.
Baker, Hinton J., United States Army.
Bennett, Michael V., Yale University.
Bennett, Theda E., Syracuse University.
Bogorad, Lawrence, Rockefeller Institute for Medical Research.
Borysko, Emil, Johns Hopkins University.
Bradford, Sarah C., McGuire Veterans Administration Hospital.
Burke, William T., University of Rochester.
Castor, Laroy N., University of Pennsylvania.
Ceas, Maria Piera, Columbia University.
Chalfin, David, Florida State University.
Covell, Geraldine M., University of Rochester.
Davidheiser, Roger, Indiana University.
DeGraff, Arthur C., New York University College of Medicine.
Epstein, Richard H., University of Rochester.
Fenn, Priscilla, University of Rochester.
Hurlbert, Robert B., University of Wisconsin.
MacLean, Elizabeth C., Mt. Holyoke College.
McNamara, Dorris M., Rockefeller Institute for Medical Research.
Menz, Leo J., St. Louis University.
Overton, Jane H., University of Chicago.
Peck, Harry D., Jr., Wesleyan University.
Punnett, Thomas R., University of Illinois.
Roslansky, John D., University of Washington.
Smith, Charles E., University of Illinois.
Stoiber, Alma Mary, University of Michigan.
Weis, Dale, Yale University.

INVERTEBRATE ZOOLOGY

Battin, William, University of Minnesota.
Bequaert, Helen M., Cornell University.
Berkowitz, Harold, Amherst College.
Blaszczynski, Henry J., St. Louis University.
Bloedel, Prentice, University of California.

Castleman, Mary, Randolph-Macon Woman's College.
Conte, Anthony R., Fordham University.
Darlington, Julian T., University of Florida.
Einhorn, Irving N., Temple University.
Flavin, John W., Brown University.
Foster, Joyce M., DePauw University.
Foster, Kendall W., Jr., Tufts College.
Friedman, Frank, New York University.
Gale, Martha G., University of Wisconsin.
Gallicchio, Vincent, University of Illinois.
Gaumer, Albert E., Purdue University.
Gilligan, Joseph I., Villanova College.
Glade, Richard W., University of Illinois.
Glassman, Edward, Johns Hopkins University.
Gojmerac, Walter L., Marquette University.
Grabowski, Casimer T., Johns Hopkins University.
Hammen, Carl S., St. John's College.
Hanzely, Joseph B., Catholic University of America.
Herzenberg, Leonard A., Brooklyn College.
Hisaw, Frederick L., Harvard University.
Huntington, Nancy, Drew University.
Kamemoto, Fred I., Purdue University.
Kohn, Vilma Lavetti, University of Michigan.
Kostyo, Jack L., Oberlin College.
Leavenworth, Martha, Carleton College.
Luyten, Mona, Oberlin College.
Lytle, Charles F., Wabash College.
McMahon, Jean, Fordham University.
McMahon, Rita Mary, Fordham University.
Maguire, Bassett, Cornell University.
Maguire, Marjorie P., Cornell University.
Marquardt, William C., University of Illinois.
Milkman, Roger D., Harvard University.
Myers, Rose M., Purdue University.
Nicely, Martha, Radcliffe College.
Pegau, Lucy B., Oberlin College.
Primack, Nathan, Purdue University.
Rawson, Kenneth S., Cornell University.
Robertson, Sally Jean, Randolph-Macon Woman's College.
Ross, Frances, Vassar College.
Rudnyansky, Bertina M., Indiana University.
Schiff, Jerome A., Brooklyn College.
Sciarra, John J., Yale University.
Silverman, Marian J., Drew University.
Small, Marcia C., University of Massachusetts.
Tanner, Wilma, Yale University.
Thomas, Hilda P., Austin College.
Walker, Jane A., Smith College.
Westfall, Jane A., Lafayette, California.
Wolk, Robert G., The City College of New York.

4. Lalor Fellows, 1952

Barton, Jay, II, Columbia University.
Boyer, Paul D., University of Minnesota.
Ciereszko, Leon, University of Oklahoma.
Connelly, Clarence M., Johns Hopkins University.

Florkin, Marcel, University of Liège, Belgium.
Freeman, Paul J., San Jose State College.
Inoué, Shinya, University of Washington School of Medicine.
Nichol, Charles A., Western Reserve University School of Medicine.
Strittmatter, Cornelius F., Harvard University Medical School.

5. Tabular View of Attendance, 1948–1952

	1948	*1949*	*1950*	*1951*	*1952*
Investigators—Total	326	344	338	303	300
Independent	183	193	198	186	172
Under Instruction	42	52	43	28	38
Library Readers	50	55	48	37	49
Research Assistants	51	44	49	52	47
Students—Total	123	128	126	124	123
Zoology	54	55	55	55	55
Embryology	29	31	29	27	23
Physiology	25	27	27	29	27
Botany	15	15	13	15	11
Ecology					7
Total Attendance	449	472	444	427	423
Less persons registered as both students and investigators	6	2			2
	443	470	444	427	422
Institutions Represented—Total	158	155	156	158	149
By Investigators	117	114	114	115	92
By Students	68	68	67	43	57
Schools and Academies Represented					
By Investigators	1	1	2	1	1
By Students					3
Foreign Institutions Represented					
By Investigators	8	6	6	8	7
By Students	4	3	2	3	2

6. Cooperating and Subscribing Institutions, 1952

Cooperating Institutions

Amherst College
Brooklyn College
Bryn Mawr College
Children's Hospital of Philadelphia
College of Mt. St. Joseph-on-the-Ohio
College of Physicians and Surgeons
Columbia University
Drew University
Duke University
Elmira College
Fordham University
Harvard University
Harvard University Medical School
Institute for Muscle Research
Johns Hopkins University
Johns Hopkins University Medical School
Eli Lilly and Company
Morgan State College
National Cancer Institute
New York University College of Medicine
New York University, Heights
New York University, Washington Square College
North Carolina State College of Agriculture and Engineering
Northwestern University
Oberlin College
Princeton University
Rockefeller Institute for Medical Research
Rutgers University
Saint Louis University
Smith College
Southwestern Medical College
State University of Iowa
State University of New York College of Medicine
Temple University
Tufts College Medical School

Tulane University School of Medicine
University of Chicago
University of Connecticut
University of Delaware
University of Illinois
University of Kansas
University of Maryland School of Medicine
University of Massachusetts
University of Michigan
University of Minnesota
University of New Hampshire
University of Pennsylvania
University of Pennsylvania Medical School
University of Rochester
University of Vermont Medical School
University of Virginia
University of Wisconsin
Vassar College
Washington University School of Medicine
Wesleyan University
Western Reserve University School of Medicine
Yale University

Subscribing Institutions

Boston Dispensary
Brown University
California Institute of Technology
Carnegie Embryological Laboratory
Central Institute for the Deaf
City College of New York
Colby College
Cornell University
Cornell University Medical College
Emory University
George Washington University
Hahnemann Medical School
Marquette University
Massachusetts General Hospital
Mount Holyoke College
National Medical Research Institute
National Science Foundation
New York Medical College
Oak Ridge Institute for Nuclear Studies, Inc.
Public Health Research Inst. of New York City
Rockefeller Foundation
St. John's College
St. Norbert College
Seaplant Chemical Corporation
Sloan-Kettering Institute
Spring Hill College
Tufts College
University of California
University of Colorado School of Medicine
University of Florida
University of Mississippi
University of North Carolina
University of Pittsburgh
University of South Dakota
Wellesley College

7. EVENING LECTURERS, 1952

June 27		
	I. TASAKI	"A survey of the development of our concept of nervous conduction."
July 4		
	H. F. BLUM	"Ultraviolet light and cancer."
July 11		
	K. V. THIMANN	"The physiology of cell elongation in plant tissues."
July 18		
	OTTO LOEWI	"From the workshop of discoveries."
July 25		
	MARCEL FLORKIN	"Some biochemical implications of adaptation in animals."
August 1		
	J. S. NICHOLAS	"The action of cells and aggregates in embryonic formation."
August 8		
	THURLOW NELSON	"Some studies of the ecology and physiology of the oyster."

August 15	
John H. Welsh	"The acetylcholine system."
August 22	
D. W. Woolley	"Antimetabolites in relation to natural processes and man's control of them."

8. Tuesday Evening Seminars, 1952

Tuesday, July 1	
Paul R. Gross	"An enzymatic study of yolk-platelet lysis."
Alfred B. Chaet	"Action of heparin preparations on cells and tissues."
L. V. Heilbrunn, Atida Halaban, Walter L. Wilson	"An attempt to influence the survival time of mice with implanted tumors."
Tuesday, July 8	
S. M. Rose	"The specific suppression of differentiation of embryonic heart, blood, and brain by the like adult tissues."
John R. Shaver	"Some studies in the initiation of cleavage in the frog egg."
Charles B. Metz, Dorothy R. Pitelka	"The structure of the neuromotor apparatus of Paramecium as revealed by the electron microscope."
Tuesday, July 15	
Robert D. Allen	"A visible response to stimulation in egg cells of the surf clam."
Daniel Mazia, Katsuma Dan	"Isolation and biochemical characterization of the mitotic apparatus of dividing cells."
J. T. Bonner	"Mitotic activity in relation to differentiation in the slime mold, *Dictyostelium discoideum.*"
Ethel Browne Harvey	"Electrical method of sexing Arbacia and obtaining small quantities of eggs repeatedly."
Tuesday, July 22	
Berta Scharrer	"Further studies of the intercerebralis-cardiacum-allatum system of insects."
Ernst Scharrer	"The neurohypophysis of elasmobranch fishes."
Carl C. Speidel	"Balantidial infection in tadpoles as recorded by cine-photomicrography."
Tuesday, July 29	
Gerald S. Bernstein	"Sperm agglutinins in the egg jelly of the frogs, *Rana pipiens* and *R. clamitans.*"
G. S. Fraenkel	"A function of the salivary glands of the larvae of Drosophila and other flies."
Francis Haxo	"Carotenoid formation in mutant strains of *Neurospora crassa.*"
Tuesday, August 5	
Andrew G. Szent-Gyorgyi	"Meromyorsin, the sub-units of myoris."

William H. Johnson	"Studies on the viscoelastic properties of smooth muscle."
C. Ladd Prosser	"Conduction in non-striated muscles."

Tuesday, August 12

Philip Person, W. W. Wainio, B. Eichel	"The partial separation of the prosthetic groups of cytochrome *b* and cytochrome oxidase from active enzyme."
Dwight B. McNair Scott	"The oxidative pathway of carbohydrate metabolism in *E. coli.*"
Jay S. Roth	"Ribonucleases."

Tuesday, August 19

Philip B. Armstrong	"Function in the developing liver."
Seymour H. Wollman, Edgar Zwilling	"Radioiodine uptake in the chick embryo thyroid gland."
Ray L. Watterson	"The glycogen body as a derivative of the avian neural tube."

9. Members of the Corporation, 1952

1. Life Members

Beckwith, Dr. Cora J., Vassar College, Poughkeepsie, New York.
Billings, Mr. R. C., 66 Franklin Street, Boston, Massachusetts.
Calvert, Dr. Philip P., University of Pennsylvania, Philadelphia, Pennsylvania.
Cole, Dr. Leon J., College of Agriculture, Madison, Wisconsin.
Conklin, Prof. Edwin G., Princeton University, Princeton, New Jersey.
Cowdry, Dr. E. V., Washington University, St. Louis, Missouri.
Dungay, Dr. Neil S., Carleton College, Northfield, Minnesota.
Jackson, Mr. Chas. C., 24 Congress Street, Boston, Massachusetts.
Jackson, Miss M. C., 88 Marlboro Street, Boston, Massachusetts.
King, Mr. Chas. A.
King, Dr. Helen D., Wistar Inst. of Anatomy and Biology, Philadelphia, Pennsylvania.
Lewis, Prof. W. H., Johns Hopkins University, Baltimore, Maryland.
Lowther, Dr. Florence deL., Barnard College, New York City, New York.
MacNaught, Mr. Frank M., Woods Hole, Massachusetts.
Means, Dr. J. H., 15 Chestnut Street, Boston, Massachusetts.
Moore, Dr. George T., Missouri Botanical Gardens, St. Louis, Missouri.
Moore, Dr. J. Percy, University of Pennsylvania, Philadelphia, Pa.
Noyes, Miss Eva J.
Payne, Dr. Fernandus, Indiana University, Bloomington, Indiana.
Porter, Dr. H. C., University of Pennsylvania, Philadelphia, Pennsylvania.
Scott, Dr. Ernest L., Columbia University, New York City, New York.
Sears, Dr. Henry F., 86 Beacon Street, Boston, Massachusetts.
Shedd, Mr. E. A.
Waite, Prof. F. C., 144 Locust Street, Dover, New Hampshire.
Wallace, Louise B., 359 Lytton Avenue, Palo Alto, California.

2. Regular Members

Adams, Dr. A. Elizabeth, Mount Holyoke College, South Hadley, Massachusetts.

Addison, Dr. W. H. F., University of Pennsylvania Medical School, Philadelphia, Pennsylvania.

Adolph, Dr. Edward F., University of Rochester School of Medicine and Dentistry, Rochester, N. Y.

Albaum, Dr. Harry G., Biology Department, Brooklyn College, Brooklyn, New York.

Albert, Dr. Alexander, Mayo Clinic, Rochester, Minnesota.

Allee, Dr. W. C., 114 Leigh Hall, University of Florida, Gainesville, Florida.

Alscher, Dr. Ruth, Dept. of Physiology, Manhattanville College of the Sacred Heart, Purchase, New York.

Amberson, Dr. William R., Dept. of Physiology, University of Maryland School of Medicine, Baltimore, Md.

Anderson, Dr. Rubert S., Dept. of Physiology, University of South Dakota, Vermillion, S. D.

Anderson, Dr. T. F., University of Pennsylvania, Philadelphia, Pennsylvania.

Angerer, Dr. C. A., Department of Physiology, Ohio State University, Columbus, Ohio.

Armstrong, Dr. Philip B., State University of New York, College of Medicine, Syracuse 10, New York.

Atwood, Dr. Kimball C., 68½ Outer Drive, Oak Ridge, Tennessee.

Austin, Dr. Mary L., Wellesley College, Wellesley, Massachusetts.

Ayers, Dr. John C., Department of Oceanography, Cornell University, Ithaca, New York.

Baitsell, Dr. George A., 54 Hill House Ave., Connecticut.

Baker, Dr. H. B., Zoological Laboratory, University of Pennsylvania, Philadelphia, Pennsylvania.

Ball, Dr. Eric G., Dept. of Biological Chemistry, Harvard University Medical School, Boston 15, Massachusetts.

Ballard, Dr. William W., Dartmouth College, Hanover, New Hampshire.

Bard, Dr. Philip, Johns Hopkins Medical School, Baltimore, Maryland.

Barron, Dr. E. S. G., Department of Medicine, University of Chicago, Chicago, Illinois.

Barth, Dr. L. G., Department of Zoology, Columbia University, New York City, New York.

Bartlett, Dr. James H., Department of Physics, University of Illinois, Urbana, Illinois.

Beams, Dr. Harold W., Department of Zoology, State University of Iowa, Iowa City, Iowa.

Beck, Dr. L. V., Dept. of Physiology and Pharmacology, University of Pittsburgh School of Medicine, Pittsburgh 13, Pennsylvania.

Beers, Dr. C. D., University of North Carolina, Chapel Hill, North Carolina.

Behre, Dr. Elinor H., Louisiana State University, Baton Rouge, Louisiana.

Bertholf, Dr. Floyd M., College of the Pacific, Stockton, California.

Bevelander, Dr. Gerrit, New York University School of Medicine, New York City, New York.

Bigelow, Dr. Henry B., Museum of Comparative Zoology, Harvard University, Cambridge, Mass.
Bigelow, Dr. Robert P., Massachusetts Institute of Technology, Cambridge, Massachusetts.
Bishop, Dr. David W., Dept. of Embryology, Carnegie Inst. of Washington, Baltimore 5, Maryland.
Blanchard, Dr. K. C., Johns Hopkins Medical School, Baltimore, Maryland.
Blum, Dr. Harold F., Department of Biology, Princeton University, Princeton, New Jersey.
Bodansky, Dr. Oscar, Dept. of Biochemistry, Sloan-Kettering Division, Cornell University Medical College, New York City, New York.
Bodine, Dr. J. H., Department of Zoology, State University of Iowa, Iowa City, Iowa.
Bodian, Dr. David, Department of Epidemiology, Johns Hopkins University, Baltimore 5, Maryland.
Boell, Dr. Edgar J., Yale University, New Haven, Connecticut.
Boettiger, Dr. Edward G., Department of Zoology, University of Connecticut, Storrs, Conn.
Bonner, Dr. John T., Department of Biology, Princeton University, Princeton, New Jersey.
Bradley, Dr. Harold C., 2639 Durant Avenue, Berkeley 4, California.
Brodie, Mr. Donald M., 522 Fifth Avenue, New York 18, New York.
Bronfenbrenner, Dr. J. J., 1703 Reynolds Road, Winston-Salem, North Carolina.
Bronk, Dr. Detlev W., Johns Hopkins University, Baltimore, Maryland.
Brooks, Dr. Matilda M., University of California, Dept. of Physiology, Berkeley 4, California.
Brown, Dr. Frank A., Jr., Department of Biological Sciences, Northwestern University, Evanston, Illinois.
Brown, Dr. Dugald E. S., Department of Zoology, University of Michigan, Ann Arbor, Michigan.
Brownell, Dr. Katherine A., Ohio State University, Columbus, Ohio.
Buck, Dr. John B., Laboratory of Physical Biology, National Institutes of Health, Bethesda, Md.
Buckingham, Miss Edith N., Sudbury, Massachusetts.
Budington, Dr. R. A., Box 954, Winter Park, Florida.
Bullington, Dr. W. E., Randolph-Macon College, Ashland, Virginia.
Bullock, Dr. T. H., University of California, Los Angeles 24, California.
Burbanck, Dr. William D., Box 834, Emory University, Georgia.
Burdick, Dr. C. Lalor, The Lalor Foundation, Lancaster Pike and Old Baltimore Road, Wilmington, Delaware.
Burkenroad, Dr. M. D., Institute of Marine Science, Port Arkansas, Texas.
Butler, Dr. E. G., Princeton University, Princeton, New Jersey.
Cameron, Dr. J. A., Baylor College of Dentistry, Dallas, Texas.
Cannan, Dr. R. K., National Research Council, 2101 Constitution Avenue, Washington N.W., D. C.
Cantoni, Dr. Giulio, Department of Pharmacology, Western Reserve University, Cleveland 9, Ohio.

Carlson, Dr. A. J., Department of Physiology, The University of Chicago, Chicago 37, Illinois.

Carothers, Dr. E. Eleanor, 134 Avenue C., East Kingman, Kansas.

Carpenter, Dr. Russell L., Tufts College, Medford 55, Massachusetts.

Carson, Miss Rachel, 204 Williamsburg Drive, Silver Spring, Maryland.

Carver, Dr. Gail L., Mercer University, Macon, Georgia.

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STRUCTURE AND FUNCTION IN THE PYLORIC CAECA OF ASTERIAS FORBESI

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In his book, *Comparative Physiology*, Scheer (1948, p. 352) makes the following statement concerning Echinodermata: "The process of digestion is not well known. Large diverticula are present in the asteroids. . . . Extracellular digestion is evident . . ., but the function of the extensive diverticula remains unsolved." A review of the literature on this subject reveals that this statement is largely justified. Although repeated attempts have been made to elucidate the mechanisms of digestion and absorption in asteroids, the conclusions drawn have been highly contradictory, and in many cases the experimental procedures are open to serious question. Indeed, it appears that no general agreement has been reached in descriptions of the histological details of the alimentary canal in starfishes.

The present report is a preliminary account of studies undertaken to clear up some of the existing confusion in the histology of the pyloric caeca, and to correlate by means of histochemical techniques the structural and functional aspects of the cells comprising the lining epithelium of these organs. Although no attempt has been made to present here an exhaustive review of all previous studies on the pyloric caeca, a brief analysis of the opinions of other investigators is included and an effort has been made to synthesize from all reliable sources a picture of the probable role of the pyloric caeca in alimentation.

MATERIAL AND METHODS

Animals used in these investigations were vigorous adult specimens of *Asterias forbesi*, ranging in size from 10 to 20 cm. in diameter. They were obtained by express from Woods Hole, maintained in 50-gallon marine aquaria, and fed regularly with portions of clam and mussel. For studies of the effect of nutritional state on the condition of the digestive diverticula, a series of animals was isolated and starved for varying periods ranging up to 8 weeks. Six to 8 weeks of starvation appeared to be the maximum which an animal of this size could withstand.

For use, one arm was removed from an animal and its paired pyloric caeca dissected out in sea water. Small pieces of the caeca were then taken and fixed in a series of fluids. These samples were subsequently processed and imbedded as appropriate for the various histological and histochemical techniques to be applied, as follows:

1) For general orientation, cell structure, and secretion granules, tissues were fixed in Zenker-formal, imbedded in paraffin by the dioxan method, and sec-

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[1] Present address.

tioned at 4 μ. These sections were stained in Mallory's phosphotungstic acid hematoxylin, Heidenhain's iron alum hematoxylin, Harris hematoxylin, or neutral gentian.

2) For demonstration of glycogen and related compounds, fixation was in Helly's fluid or in cold Rossmann, followed by treatment of paraffin sections by the Hotchkiss-McManus periodic acid-Schiff routine. Control slides exposed to the action of a buffered solution of malt diastase were used for differentiation between glycogen and other Schiff-positive substances.

3) For the recognition of mucin and other similar compounds (acid polysaccharides), paraffin sections of tissues fixed in buffered Orth's fluid or in basic lead acetate-formaldehyde were stained overnight in very dilute aqueous solutions of toluidine blue and dehydrated through 95% and absolute alcohol. This routine brings about a metachromatic staining of acid-polysaccharide elements. Representative sections of this material were also treated by the periodic acid-Schiff method.

4) For the localization, and, in part, the characterization of lipids, tissues were fixed in Baker's formal-calcium or formal-saline. For general recognition of lipids, tissues fixed in formal-saline, and soaked at 60° C. for 24 hours in 5% potassium dichromate, were imbedded in gelatin, sectioned on the freezing microtome, colored with Sudan black, and counterstained with Mayer's carmalum. For the localization of triglycerides, similarly fixed, postchromed, imbedded, and sectioned material was stained in 1% Nile blue A and differentiated in 1% acetic acid (Cain, 1947a). Phospholipid was investigated in material fixed in formal-calcium and treated by Baker's acid-hematein method, controlled by the pyridine-extraction test applied to adjacent samples fixed in weak Bouin's fluid (Baker, 1946).

5) For the demonstration of alkaline phosphatase activity, tissues were fixed in ice-cold acetone and imbedded in paraffin. Four-micron sections were incubated at 37° C. in a solution of sodium-beta-glycerophosphate and then carried through the routine of Gomori, in which sites of phosphatase activity are visualized as brown or black deposits of cobalt sulfide. Control sections were processed simultaneously, omitting only the substrate-incubation step.

It is a pleasure to acknowledge the capable technical assistance of Dorothy T. Clarke, Research Assistant in Biology at Brown University, in the conduct of a large part of this work.

Observations

A. General structure of the pyloric caecum

Each diverticulum constitutes one member of a pair in each arm, representing branches produced by the bifurcation of the single pyloric duct proceeding into the arm from an angle of the pyloric stomach. Each diverticulum is suspended by a pair of longitudinal mesenteries, enclosing between them a part of the epigastric coelom. The sheets comprising the mesenteries are continuous with the somatic mesothelium lining the body wall, and with the splanchnic mesothelium covering the diverticulum.

The pyloric duct continues the length of the caecum, forming a central tubular cavity known as Tiedemann's diverticulum. Serial outpocketings from the lateral walls of this tube form a row of pouches, whose cavities communicate broadly with that of the central passageway. By a series of vertical folds, the walls of these primary pockets form secondary bays, branching in a radial pattern. The secondary bays may also be subdivided, by both vertical and horizontal folds in their walls, to form the ultimate blind cavities of the caecum.

The wall of this complex organ is relatively simple, consisting of layers which may be described as follows:

a. As noted above, the organ is covered by a peritoneal layer. This is composed of small, cuboidal cells and is stretched thin over convexities but thrown into folds in markedly indented areas. Each cell of this layer bears a single, long flagellum.

b. Layers of muscular, nervous, and connective tissue fibers, variously represented, lie under the splanchnic peritoneum (for details, see Hamann, 1885, and Chadwick, 1923).

c. The epithelial lining is chiefly responsible for the thickness of the wall. It is composed of a single layer of extremely tall, slender cells forming a pseudostratified columnar epithelium, resting upon a basement membrane. This layer contains several distinct cell types, described below in detail. Speaking generally, the free ends of the epithelial cells are covered by a thick brush border, and each cell bears a single long flagellum. The height of the epithelium varies tremendously, being least in the angles of evaginated folds in the wall. At these points the lumen appears to extend almost to the basement membrane, while in the walls between these thin spots the epithelium may be from 75 to 90 μ in height.

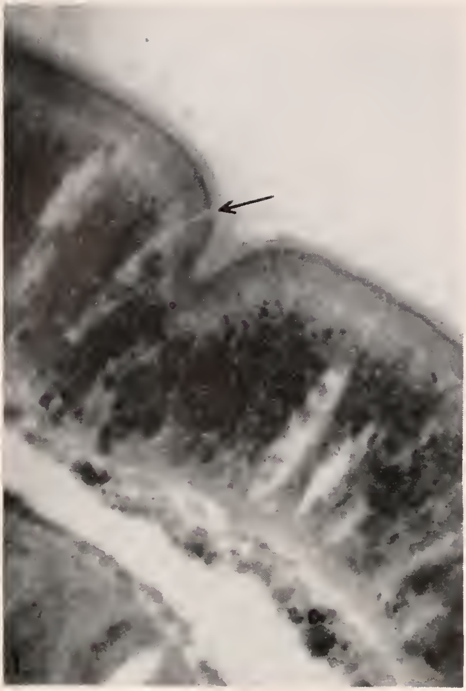
The following section will be concerned with elucidation of the cellular constituents of this lining epithelium and the characterization of the various cell-types represented, in terms of their histological and histochemical details.

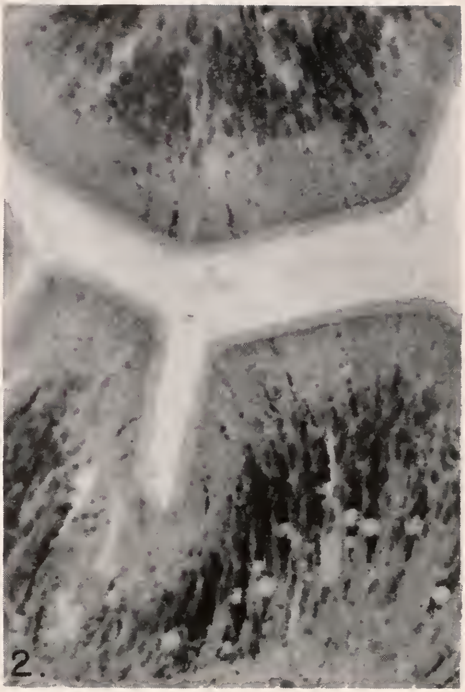
B. Organization of the lining epithelium

The cells of which the epithelium is composed fall into the following categories: (a) special current-producers; (b) storage cells; (c) secretory or zymogen cells; (d) mucus gland cells.

a. Special current-producers are structurally highly adapted to the production of currents in the lumen of the diverticulum but apparently have no other special function. These cells are characteristic of the lining of Tiedemann's diverticulum and of the oral and aboral walls of the primary pockets arising from it. The structural details of these cells vary with their location, and apparently with the degree of crowding to which they are subjected. In the aboral wall of Tiedemann's diverticulum, between the attachments of the mesenteries, they are moderately tall, remarkably slender, and very closely packed (Fig. 9). In these cells the nuclei are long and spindle-shaped, measuring about 8 $\mu \times 2$ μ. They are granular, and each contains a small nucleolus. In other regions the current-producers are taller, less crowded, with densely-staining oval nuclei. In all, the nuclei lie at various depths in the epithelium, from a point near the basement membrane to within a short

PLATE I





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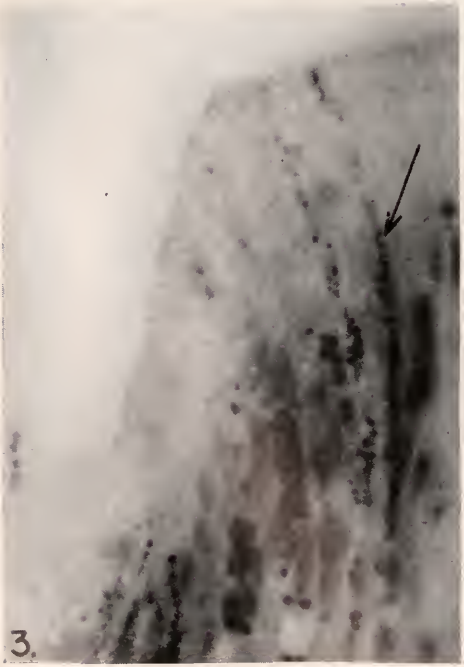

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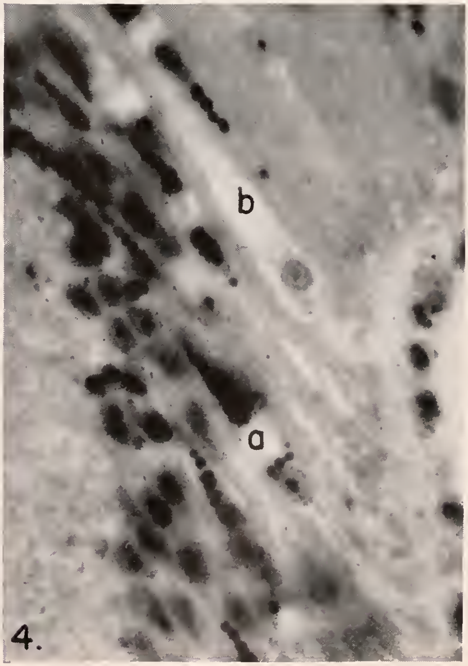

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distance of the free edge (Fig. 1). The cells are broadest in the nuclear region, and from the basal end of this area in each cell a tapering fibrous process extends to the basement membrane. Although this is difficult to ascertain, each cell appears to be represented at the surface of the lumen, where it bears a conspicuous brush border and a single long flagellum, ½ to ⅓ as long as the cell. The flagellum springs from a prominent blepharoplast lying just under the brush border and sending downward into the cytoplasm a stout chromophilic strand.

In all areas of which these cells are characteristic, mucus gland cells are abundant (Figs. 1 and 10).

b. Storage cells, in the lateral walls of the primary pockets and throughout the lining of the secondary bays and ultimate branches of the caeca, replace the current-producers as the most numerous class. These cells average about 5 μ in diameter and vary in height from 15 μ to a maximum of about 90 μ, depending upon their location and upon the size of the animal. Their nuclei are broadly oval or subspherical and generally stain densely and uniformly, except for the small nucleolus. The nuclei occupy a broad band limited approximately to the middle third of the cells. Each cell bears, again, a single flagellum originating in an apical blepharoplast and extending well beyond the brush border. Sections cut tangentially to the surface of the epithelium in these regions show that the cells are roughly polygonal in cross-section, and that the blepharoplasts, one in each cell, do not occupy the center of the apex but lie very near the cell membrane to one side (Fig. 6) and may be attached to it. Close study of sections passing longitudinally through this region gives the impression that the blepharoplast gives rise to one strand running in contact with the cell membrane and to another passing more directly downward into the cytoplasm (Fig. 3).

In a normal, well-fed animal, these cells contain abundant deposits of lipids, lying both above and below the nucleus in the form of moderate-sized droplets. The lipid droplets are larger and more numerous in the basal portions of the cells; above the nuclear region they become smaller and more sparse and are usually lacking from the distal quarter of the cell (Fig. 7). The Nile blue technique reveals that triglycerides predominate in the constitution of these lipid deposits; no elements have been recognized in the storage cells which are sudanophilic and stain other than pink with Nile blue. The acid hematein test has failed to reveal phospholipid deposits.

Figures 1 through 6 represent sections of material fixed in Helly's fluid, sectioned at 4 to 6 μ, and stained in Mallory's phosphotungstic acid hematoxylin. In all cases, the indicated magnification is approximately that of the figure as it appears here.

PLATE I

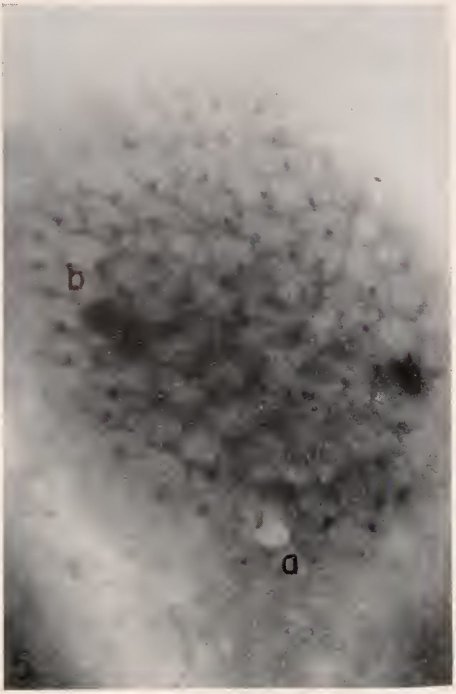
FIGURE 1. Special current-producing cells, moderately crowded region. Lumen above, perivisceral coelom below. Note row of blepharoplasts, brush border, flagella. Large clear areas are mucus goblets; mucus stoma indicated by arrow. 500 ×.

FIGURE 2. Storage and secretory cell areas, from same slide as Figure 1. Note vacuoles surrounded by secretory granules. 500 ×.

FIGURE 3. Part of same region, 1200 ×. Note extent of granule-train indicated by arrow; also divergent chromophilic strands from blepharoplasts.

FIGURE 4. Secretory cell region. At *a*, note relationship between nucleus and secretory products; at *b*, part of a mucus gland cell with empty mucus vacuole. Peritoneum at right; lumen off left. 1200 ×.

PLATE II



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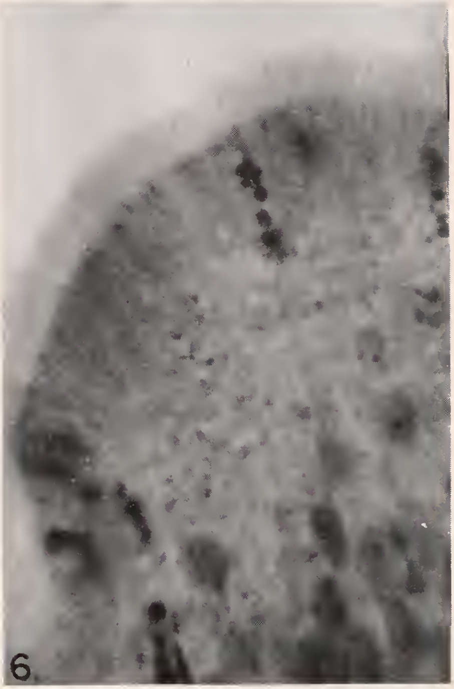

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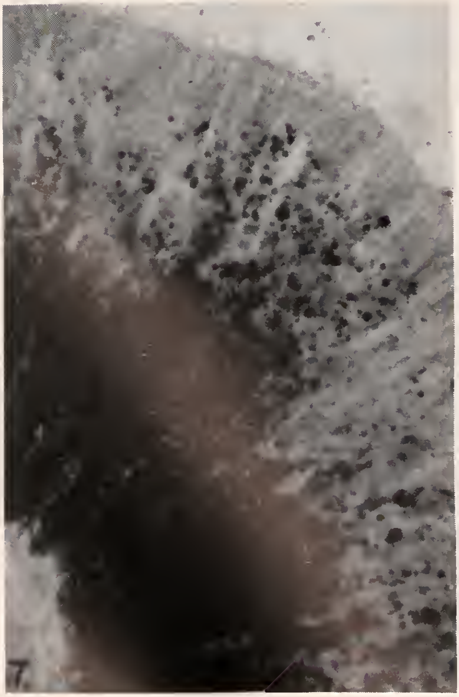

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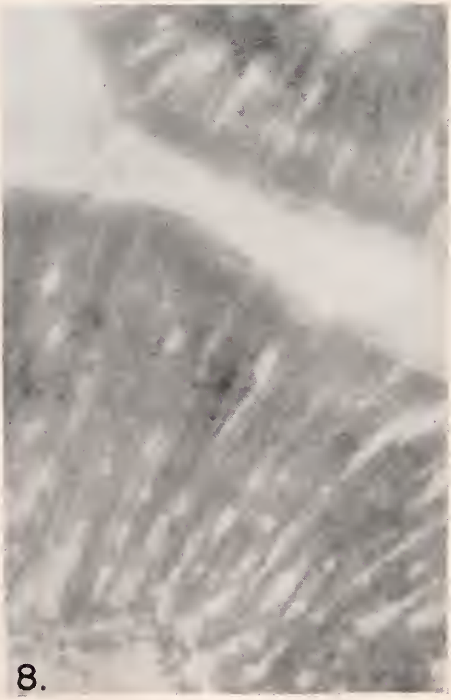

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The ground-cytoplasm of the storage cells also contains a small amount of glycogen, in addition to more abundant stores of a related compound giving a positive reaction with the Hotchkiss-McManus technique even after prolonged exposure to saliva or malt diastase. This positive reaction is slowly abolished by the action of pancreatin. The substance does not stain metachromatically with toluidine blue. It is presumably a polysaccharide-protein complex. The small deposits of glycogen, and particularly the copious amounts of this unknown compound, have a distribution in the cell similar to that of the lipids; *i.e.,* they are most abundant in the region between nucleus and basement membrane, become scanty above the nucleus, and are absent from the distal ends of the cells (Fig. 9). Storage cells frequently contain large globules, giving the reactions of the unknown substance, scattered in the region above the nucleus (Fig. 9).

In an animal subjected to prolonged starvation, all of these reserves disappear from the storage cells. Glycogen, never abundant, is used first, and its disappearance is followed by a gradual decrease in demonstrable lipids (Fig. 8). The carbohydrate-protein compound also vanishes in the course of 6 to 8 weeks' starvation.

The greenish-yellow color of the pyloric caeca appears to be associated chiefly with the storage cells. Each of these cells contains numerous granules of a greenish pigment, limited to its distal portion. The pigment granules are not sudanophilic but frequently stain after prolonged treatment with phosphotungstic acid hematoxylin.

c. Secretory or zymogen cells are never encountered in the regions occupied by special current-producing cells but are numerous among the storage cells, which they resemble in some respects. Their nuclei are similar in size and shape to those of storage cells but have a more granular, less homogeneous appearance. The nucleus occupies the broadest region of the spindle-shaped cell, and in neighboring cells this swelling may be higher or lower, presenting a staggered arrangement of the nuclei in these closely-packed cells.

The outstanding feature of the secretory cells is their content of secretory granules, presumably zymogenic (Figs. 2, 3 and 4). These granules lie in clumps in the expanded region adjacent to the nucleus, both above and below the nucleus, and may extend in one or more rows completely to the free end of the cell. Very active cells also show dense aggregations of granules in their basal ends. Associated usually with the supranuclear granule clump appears a clear vacuole; very rarely, but occasionally, this vacuole may lie below the nucleus. The granules pass upward through the cell, crowding past the nucleus, and may often be observed as if on the

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PLATE II

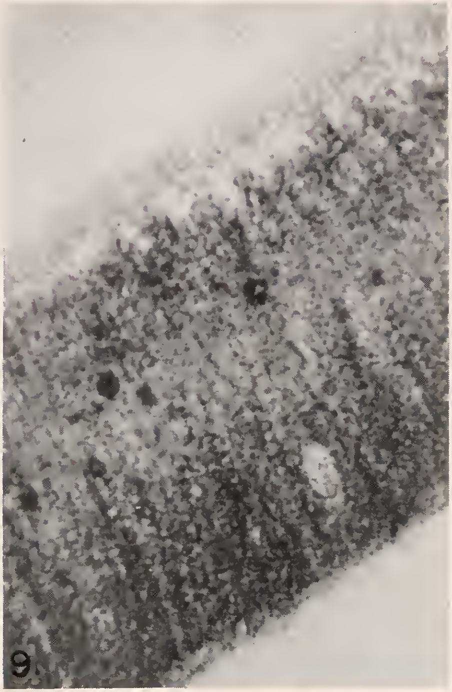
FIGURE 5. Distal ends of storage and secretory cells, section tangential to surface of epithelium. Note shape of cells, eccentric positions of blepharoplasts and their apparent attachment to cell membrane. At *a*, mucus stoma; at *b*, secretory cell. 1500 ×.

FIGURE 6. Distal ends of storage and secretory cells, longitudinal section. Note brush border, blepharoplasts, chromophilic strands, flagella. 1500 ×.

FIGURE 7. Storage cell area, showing lipid deposits. Note concentration of droplets toward basal ends of cells. Formal-saline; frozen section colored with Sudan black, counterstained with carmalum. 500 ×.

FIGURE 8. Storage cell area in animal after 8 weeks' starvation. Preparation exactly as in Figure 7; note exhaustion of stored lipids. 500 ×.

Plate III



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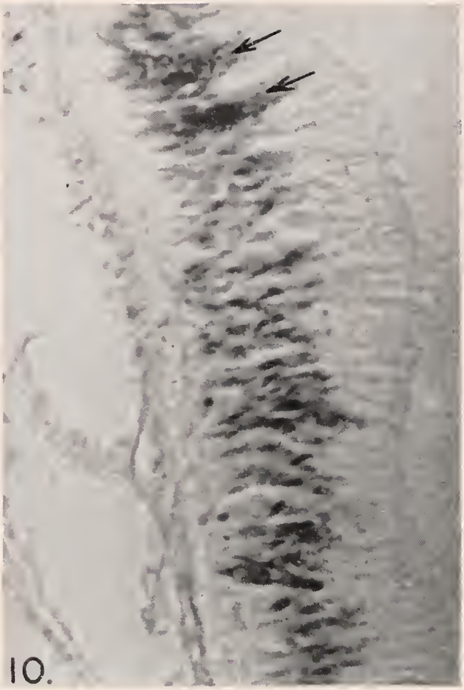

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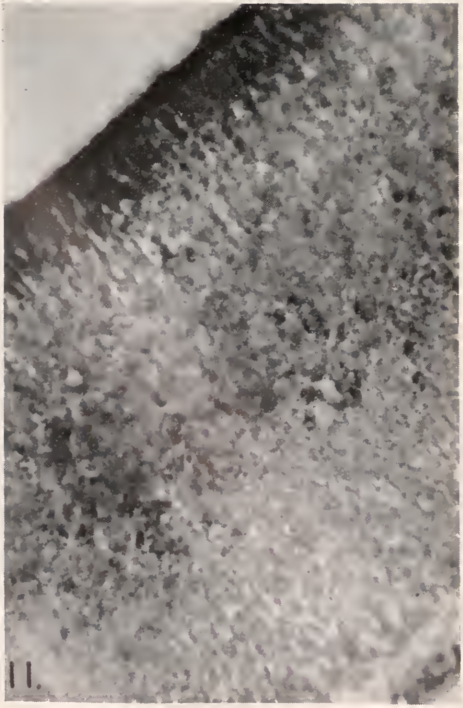

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point of being extruded at the free end. The vacuole also appears to move upward through the cell with the granular clump but has not been observed near the apical end.

The vacuoles and secretory granules are well preserved by all effective cytoplasmic fixatives, and the granules are easily demonstrable by such staining methods as Heidenhain's iron alum hematoxylin, Mallory's phosphotungstic acid hematoxylin, and the neutral gentian technique. In frozen sections of material prepared for fat staining, they appear as clear, somewhat refractile spherules. Under the action of fixatives containing acetic acid they break down, and in sections of such material the secretory cells appear to contain only a rather densely granular, basophilic cytoplasm.

To date it has been impossible to establish the morphology of the distal ends of secretory cells. Unless it contains secretory granules the free end of a cell cannot with certainty be related to an underlying granule-filled nuclear region. The crowding of the cells is such that even in thin sections several storage and secretory cells are usually superimposed. In an unmistakable secretory-cell apex, the cytoplasm is so packed with granules as to obscure details (Fig. 5).

d. Mucus gland cells, as indicated above, are most numerous in regions lined by special current-producers. They also occur, less frequently, scattered among the storage and secretory cells. Mucus cells invariably extend almost to the basement membrane of the epithelium, and the nuclei of these cells are basally located. The overlying space is filled with mucus which usually expands the cell to a diameter many times that of the neighboring cells (Figs. 1 and 4). Above this expanded portion, at a variable distance from the base, the cell narrows and finally opens into the lumen of the caecum at a restricted stoma, devoid of any brush border or other structural specialization (Fig. 1). In fact, one may question whether the mucus cell as such comprises anything more than the basal portion of the gland; it is conceivable that the secretion is released from the gland cell at some lower point and exudes through a flask-shaped canal formed by the walls of adjacent cells.

In teased living preparations examined in sea water the secretion of the mucus glands consists of clumps of large globules. In fixed, sectioned preparations the globules are collapsed, and the secretion has the form of films or strands traversing the cavity filled in life by the globules. The material composing these strands reacts vigorously with the Schiff reagent after treatment with periodate and exhibits a strong, purplish metachromasia with dilute toluidine blue (Fig. 10). After its

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PLATE III

FIGURE 9. Storage cell area. Rossmann; Hotchkiss-McManus technique. Note basal concentration of Schiff-positive material, most of which is not removed by diastase; empty apical regions; large scattered globules. Coelom below, peritoneum missing. 500 ×.

FIGURE 10. Mucus gland cells in special current-producing area near mesentery attachment; *cf.* Figure 1. Note length of flagella (right), densely crowded cells. Arrows indicate two large metachromatically-staining mucus glands. Basic lead acetate-formaldehyde; dilute toluidine blue. 500 ×.

FIGURE 11. Storage cell area, alkaline phosphatase activity. Note concentration of activity at brush border and in apical areas; *cf.* Figures 10 and 12. Cold acetone; Gomori technique. The carmalum counterstain is responsible for most of the dark appearance of the lower areas. 500 ×.

FIGURE 12. Section adjacent to that shown in Figure 11, treated similarly but incubation with substrate omitted. Nuclei stained with carmalum. 500 ×.

release from the stomata, the secretion spreads thinly and generally over the free ends of the epithelial cells and by its characteristic reactions can be demonstrated about the fibers of the brush border.

In terms of the epithelium as a whole, alkaline phosphatase activity appears to be largely limited to the regions occupied by the storage cells. It is lacking or weak in the areas lined by the current-producing cells. This enzymatic activity is very strong at the free ends of the cells, centering about the region of the brush border and in the distal areas previously seen to be free of stored reserves (Fig. 11). It is weak or lacking in the deeper portions of the epithelium.

Discussion

It comes as a surprise to discover that both inner and outer epithelia of the alimentary canal are provided with vibratile organelles which from their length and distribution must properly be termed flagella, instead of cilia. Most previous investigators in describing these epithelia have referred to them as ciliated. For example, Hamann (1885) cites *"die heftige Wimperung"* of the caeca, and (p. 62) specifically likens the points of attachment of these organelles to those of the cilia on the molluscan gill. Van der Heyde in both the English (1922) and French (1923) versions of his dissertation speaks of cilia, as does Chadwick (1923). In justice to Hamann it should be stated that his observations of the current-producing organelles were largely accurate; he writes (p. 62) (here in connection with the cardiac stomach): *"Die Epithelzellen des Magendarmes sind lange cylindrisch bis haarfeine Zellen, welche je eine Wimper tragen."* Correct as to number, his use of the word *Wimper* is not in accordance with modern usage. On the other hand, Schneider (1902) describing the same elements uses the word *Geissel*, which in translation would be more likely to appear as "flagellum" than would *Wimper*. Authors concerned more with the physiology than with the morphology of Asterids have been unanimous in their use of the term "cilia" (Gemmill, 1915; Irving, 1924, 1926; Budington, 1942). While one does not wish to argue about words *qua* words, it appears only proper to bring the description of these structures in Asterias into line with consistent modern terminology.[2]

The histology of the digestive tract in various Asterids has been the subject of several investigations varying greatly in thoroughness, accuracy, and interpretation of facts. Hamann (1885) describes in some detail the mucus gland cells of the pyloric caeca, referring to them as *Becherzellen*. He concludes that they form originally at the base of the epithelium as small cells which move toward the free border, increasing in size and finally establishing stomata at the lumen border. The remainder of the cell population of the epithelium he does not analyze, grouping them in a brief discussion simply as "epithelial cells," each with one or more *Wimpern* (*"das erste scheint die Regel zu sein"*), and containing a finely-granular cytoplasm. Considering the staining methods used by Hamann (acetocarmine, Böhmer's hematoxylin) it is not surprising that he found no trace of the secretory cells with their abundant coarse granules.

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[2] In this connection, Hyman (1940, footnote, p. 375) comments upon the failure of workers on Coelenterates to distinguish between cilia and flagella and is herself careful to make this distinction in every justifiable case. It should also be mentioned that upon closer study of developmental stages of *Asterias forbesi* in living and freshly-fixed material, as well as in thin paraffin sections, it is apparent that the general "ciliation" of the blastula and gastrula, and the frequently cited "ciliated bands" of the larva, are actually formed by cells bearing each a single, long, vigorous flagellum. The illustrations, and in part the text, of Korschelt and Heider (1895) appear to bear out this interpretation. If, as seems likely, flagellated rather than ciliated epithelia are the rule among Echinodermata, this peculiarity is shared among very few of the animal phyla (Porifera, Coelenterata in part, scattered instances in vertebrate tissues) and makes a close comparison of echinoderm larvae with the annelid-mollusk trochophore even more difficult.

Schneider (1902) was apparently the first to observe these secretory cells. He terms them *Eiweisszellen* and describes their coarsely-granular secretion-product as moving up to the free ends of the cells in two or three rows. His description of the mucus gland cells is somewhat more accurate than Hamann's, and he recognizes also a third cell-type, *Nährzellen,* otherwise undifferentiated. It is significant that Schneider does not ascribe a storage function to any of these types and considers the pyloric caeca as organs of secretion and possibly absorption.

MacBride (1906) writes simply that the cells forming the walls of the pyloric sac and its appendages are tall, narrow, cylindrical cells crowded with granules which appear to be of the nature of digestive ferments.

Chadwick (1923) first established the regional differentiation of cell-types characteristic of the Asterid pyloric caeca. "At two points which are practically in the median line of each caecum," he writes (p. 22), "the structure of the wall differs from that which forms the lateral sacculi. One of these is that portion of the wall of the caecum which lies between the points of attachment of the two mesenteries by which it is suspended from the aboral wall of the ray, the other is a deep fold which traverses its free oral face. In the latter the nuclei of the epithelial cells are much more densely crowded than are those of the sacculi, and in sections they appear as a fairly broad band. These cells bear especially long and powerful cilia." Here Chadwick is clearly referring to the cells which I have termed "special current-producers." Chadwick also describes and figures the vacuoles in the secretory cells, mistaking them for small mucus glands; he does not mention the secretory granules which always surround them.

The latest attempt to establish the histological details of the pyloric caeca of *Asterias* was that of Dorman (1928). Alone among those who have worked with this tissue, Dorman describes the epithelium as consisting of several layers of cells, to which by certain histochemical tests he ascribes different functions. According to this description, "hepatic cells" lie in two or three rows near the basement membrane and are rich in fat and glycogen; "pancreatic cells" occupy the inner part of the wall and are described as "exceedingly granular" and said to contain some glycogen. The remaining cells are "interstitial cells," present in large numbers, some squamous and some spindle-shaped.

Aside from the fact that such an attempt to perpetuate the "hepato-pancreas" concept of invertebrate midgut diverticula seems unnecessary, this description is widely at variance with the results of the present study. However, the sources of Dorman's confusion are evident. The architecture of this epithelium is such that a section passing through it at an angle to the long axes of the tall columnar cells could easily be interpreted as showing several layers of cells; in such a section no single cell would actually appear in its entirety. The illusion of layering is heightened by the variation in the location of nuclei in these cells. Moreover, the stratification of the carbohydrate and lipid reserves in these tall cells, so as to fill their basal ends while leaving the apices relatively empty, would in a poorly-oriented section lead to exactly the conclusion drawn by Dorman. Unfortunately, the single figure of this tissue furnished by Dorman is of no assistance in substantiating this explanation.

Several investigators have reported results of experimentation and observation relating to the functions of the pyloric caeca in Asterids. The question of their role in digestion and absorption was long debated, Frenzel (1892) maintaining that their sole function was to secrete enzymes which passed into the stomach and acted there upon ingested food. Frenzel convinced himself that no food ever enters the caeca, and in order to account for the fat deposits observed in the cells of the caeca was forced to postulate that these cells absorb from the coelomic fluid fats previously digested in the stomach. Opposite conclusions were reached by Chapeaux (1893), who found no absorption in the stomach and demonstrated (a) that carmine grains fed along with fibrin were subsequently found in the caeca, and (b) that oil-fed animals later showed fat-droplets stored in the cells of the caeca. The presence of a variety of digestive enzymes in the caeca was unquestioned, having been detected in extracts both by Chapeaux and by Fredericq (1878).

Stone (1897) and van der Heyde (1923) were unable to demonstrate detectable amounts of glycogen in the walls of the caeca by chemical analysis. This fact, together with a series of experiments involving perfusion of the digestive tract with iron saccharate, ammonium carminate, and olive oil, all of which were later found in the caecal epithelium, led van der Heyde to the conclusion (p. 139) that "this liver, without doubt, like the livers of so many other invertebrates, is principally and primarily an organ of absorption."

That more is involved in the function of the caeca than simple absorption of foods previously digested in the stomach is clearly indicated by the experiments of Irving (1926). In this work Irving perfused surviving isolated caeca of the starfish *Patiria miniata* with solutions of gelatin in sea water and was able to demonstrate a progressive rise in the non-protein nitrogen levels in the sea water in which these caeca were immersed. This technique, well-controlled, yielded unquestionable evidence that the caeca can digest proteins, and that the products of digestion are passed through the epithelia into the surrounding medium. In the intact animal, this medium would be the fluid of the perivisceral coelom, which is chiefly responsible for the distribution of absorbed nutrients throughout the body.

The work of Dorman (1928), apparently misguided in its morphological aspects, still indicates that reserves are stored in the cells of the caecal epithelium. This conclusion is justified also on the basis of the present investigation. Glycogen, another polysaccharide complex, and lipids are all demonstrable in the storage cells of the caeca. The conclusion that these represent stored reserves rests upon the evidence, presented above, that they disappear upon starvation. Starvation for one or two weeks is sufficient to demonstrate a detectable decrease in these reserves, and prolonged starvation results practically in their disappearance. This disappearance is not, moreover, the result of general moribundity of the tissue; sections of the pyloric caeca after 8 weeks of starvation showed a completely normal epithelium with no apparent decrease in the secretory activity of mucus gland and zymogen cells.

In connection with the oil-perfusion experiments of Chapeaux and of van der Heyde, which (together with the absence of glycogen) largely motivated van der Heyde's assumption of absorption as the primary function of the caeca, one looks in vain in their accounts for any indication that they examined for fatty deposits the caeca of animals which had not been fed on oil. As indicated by the results of the present study, such a simple control procedure would undoubtedly have shown that the caecal epithelium of any normal animal in a reasonably good nutritional state contains abundant stores of lipid. Lacking such controls, their arguments lose force.

It is clear, however, that absorption must occur before reserves can be stored; the abundance of alkaline phosphatase activity at the free borders of the storage cells furnishes another indication of the importance of the pyloric caeca as organs of absorption.

From the work of Cain (1947b) and others, one would expect to find deposits of histochemically-detectable phospholipids in connection with the Golgi element and mitochondria of these epithelial cells. As indicated above, however, the acid-hematein test, controlled by the pyridine-extraction test, has given consistently negative results with this tissue. It should be pointed out that the classical cytology of the epithelial cells of the pyloric caeca has apparently never been described, and the Golgi element and mitochondria of these cells have not as yet been recognized.

In summary, considering the evidence from all sources, the functional role of the pyloric caeca appears manifold. Particulate, partially-digested food is drawn from the stomach along Tiedemann's diverticula by the action of the strong flagellated cells localized here. Side currents (see Irving, 1924, and Budington, 1942) carry the particles into the lateral branches and maintain a circulation; a variety of enzymes from the secretory cells completes the digestion of the food (for a recent account of the proteolytic enzymes involved see Sawano, 1936); the products of digestion are absorbed by the storage cells, where some are elaborated into reserves of polysaccharide and lipoid nature while others pass directly through the wall of the caecum into the perivisceral coelomic fluid for distribution.

All studies concerned with the enzymatic complement of the caeca agree that proteins and carbohydrates are digested here, but a question remains as to whether fats are actually digested, or whether they are strongly emulsified and pass into the cells as minute droplets. Chapeaux (1893) held that no lipase was present and postulated that fat digestion was a function of the free amoebocytes of the coelomic fluid, which engulfed and digested fat droplets passed unchanged through the wall of the pyloric caeca. This interpretation of the role of amoebocytes was disproved by van der Heyde (1923), without, however, any evidence that lipolytic activity occurs in the caeca. The fat-storing proclivities of the storage cells indicate that they handle large amounts of lipid, and an investigation of the mechanism involved would be of considerable interest.

The significance of the apparently two-fold secretory products of the zymogen cells is also unknown. Many invertebrates secrete digestive enzymes in the form of fluids enclosed in vacuoles and do not form zymogen granules. It remains an interesting possibility that the secretory cells of the Asterid pyloric caeca, known to elaborate a variety of enzymes, secrete their various products in different forms.

Summary and Conclusions

Histological and histochemical investigations of the pyloric caeca in *Asterias forbesi* reveal that their walls are composed of an outer splanchnic peritoneum made up of small, flagellated, cuboidal cells; layers of muscular, nervous, and connective tissue fibers variously developed; and an inner epithelium generally composed of very tall, slender, flagellated cells bearing a dense brush border. Certain special areas (Tiedemann's diverticula) are lined with closely-packed cells termed "special current-producers," functioning to maintain directed movements of the contents of these central tubular cavities. Interspersed among these cells are numerous mucus gland cells. The walls of the lateral outpocketings of the central cavity are lined by an epithelium consisting of (a) secretory cells, producing conspicuous secretory (zymogen) granules and another product contained in small, clear vacuoles; and (b) storage cells, containing abundant lipids (almost entirely neutral fat), glycogen, and a polysaccharide-protein complex resistant to diastatic digestion. Mucus gland cells are less numerous in these areas. "Special current-producers" do not contain appreciable amounts of reserve substances, and the regions of the epithelium of which they form the lining do not show the vigorous alkaline phosphatase activity characteristic of the free border in the storage-cell areas. Starvation for 6 to 8 weeks results in a complete disappearance of all reserves but does not impair the secretory activities of mucus gland cells or of zymogen cells.

Previous works on the structure and functions of the Asterid pyloric caeca are summarized, and from all sources of evidence it is concluded that these organs function in digestion of food which passes into them from the stomach, in absorption of the products of digestion, and in storage of reserves. Transfer of nutrients to the coelomic fluid, for distribution throughout the body, is also a feature of their activities.

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MARINE BIOLOGICAL LABORATORY

REDOX INDICATOR PATTERNS IN RELATION TO ECHINODERM EXOGASTRULATION.

I. OXIDATION PATTERNS

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In a recent paper on gastrulation in the echinoid, *Dendraster excentricus,* following trypsin treatment, A. R. Moore (1952, p. 45) found that gastrulation occurred, even though the hyaline membrane had been removed by trypsin. Concerning this result he said, "This proves that the tensile force of the outer membrane is not an agent in gastrulation. The evidence leads to the conclusion that the process of infolding is the result of properties inherent in the vegetal plate" (Moore, 1952, p. 45). This conclusion, together with certain data concerning redox indicator patterns in echinoderm development, suggested to the present writer that further investigation of indicator patterns, particularly in relation to exogastrulation, might give some evidence concerning conditions which determine or are associated with entogastrulation and exogastrulation. The earliest observations on echinoderm indicator pattern concerned only patterns of intracellular reduction by decrease of oxygen content in the external medium after staining by oxidized "vital" dyes. As the title indicates, the present paper concerns only oxidative patterns: the data concerning reduction patterns are not complete, particularly as regards exogastrulation. It is hoped that, not only the earlier, but also more recent data concerning reduction patterns may be considered in a later paper. In the following data concerning oxidative patterns, particular attention is given to a feature of these patterns, not fully recognized in earlier work in consequence of the preoccupation with reduction patterns.

MATERIAL AND METHODS

The echinoids, *Dendraster excentricus* and *Strongylocentrotus purpuratus,* and the asteroid starfish, *Patiria miniata,* have served as material, either at the Hopkins Marine Station, Pacific Grove, or at Palo Alto after transportation of the animals under slight refrigeration, and development in most cases at temperatures ranging from 18 to 20° C., or in some lots at approximately 13° C., or even 8–10° C. with *Dendraster,* during earlier stages. In *Dendraster* and *S. purpuratus* good fertilizations were obtained for three days after transportation to Palo Alto with *S. purpuratus* and for five days with *Dendraster* from animals kept in a refrigerator at 10–15° C., covered by seaweed or wet paper toweling and with a small amount of water in the container. Starfish material for the present purpose was fertilized at Pacific Grove and brought to Palo Alto at once. *S. purpuratus* is more sensitive to environmental conditions than the other forms used.[1]

For intracellular oxidation a modification of the indophenol or Nadi reaction with very low concentrations of the two reagents, para-amino-dimethyl aniline (dimethyl-paraphenylene diamine) and α-naphthol as described repeatedly in detail in earlier papers (*e.g.,* Child, 1944, 1953b). Intracellular oxidation of the two reagents is catalyzed by an oxidase, regarded by some as cytochrome oxidase. With the low concentrations of the reagents and without the necessity of using alkali to dissolve the naphthol, the material is not killed at once and motile stages remain active until intracellular concentrations of indophenol become high. This modification of the Nadi reaction makes directly visible very slight regional differentials even more clearly in most cases than the catalyzed intracellular oxidations of dye solutions reduced by non-toxic concentrations of sodium hydrosulphite. This is largely due to the brilliant blue color of intracellular indophenol, even in very low concentrations.

In addition to the indophenol reaction various dyes, chiefly diazine green, methylene blue and in some cases Nile blue sulphate, reduced in external solution by sodium hydrosulphite ($NaHSO_2$ or $Na_2H_2S_2O_4$) were used. Minute amounts of hydrosulphite, fractions of a milligram, are sufficient to reduce one ml. of methylene blue 1/10,000 and the lower concentrations used of diazine green and Nile blue sulphate. This reducing agent has great advantages over the highly toxic reducing agents used in earlier work with redox indicators: those sometimes led to errors in results by retarding or completely inhibiting reduction in the most sensitive regions. Hydrosulphite is not appreciably toxic in concentrations and with exposure periods far above those required for dye reduction. Intracellular oxidation will occur in completely reduced external hydrosulphite dye solution if there is no great excess of hydrosulphite. The indophenol reaction and intracellular dye oxidation have been used repeatedly and results observed in many hundreds of individuals during the breeding seasons of most years from 1947 on.

Figures are essentially optical sections along the polar axis. Different magnifications are used in the three species. The egg and developmental stages of *S. purpuratus* are considerably smaller than those of the other forms and those of the starfish are larger than the *Dendraster* stages. Sizes of figures do not indicate the actual differences in size in the three forms but have been chosen chiefly in order to show the gradient patterns clearly. Figures of *S. purpuratus* are considerably more, those of the starfish less, magnified than the actual differences in size would require for representation. Intracellular indophenol reaction and dye oxidation are indicated by shading and by arrows pointing from regions of more rapid to those of less rapid reaction. Arrows external to the inhibited ectodermal regions of exogastrulae indicate only differentials along the polar axis without reference to differentials in the cell-wall. In the more extreme types of exogastrulae the ectoderm is more inhibited than entoderm and these differentials are slight and are indicated by short arrows, or they may be entirely absent. In all except one figure the entodermal differentials are indicated by shading.

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[1] The kindness of the Director and staff of the Hopkins Marine Station in providing material and laboratory facilities, and in many cases for transporting the animals to Palo Alto, is gratefully acknowledged.

Earlier Data Concerning Oxidation Patterns

The sand dollar, *Dendraster excentricus,* was the material for the earliest observations on oxidation patterns, and the indophenol reaction was used. Only the more important features of these patterns were discussed and these only briefly (Child, 1941b). In later cleavage stages and in blastulae of normal[2] development, the reaction was distinctly differential, decreasing in rate basipetally from the apical[3] region. In the gastrula and probably earlier, a ventrodorsal reaction gradient also became visible with ventral ectodermal region oxidizing more rapidly than dorsal. Within the blastocoel, mesenchyme apparently reacted more rapidly than entoderm, but with progress of invagination an entodermal gradient, decreasing basipetally from the tip of the archenteron, developed and rate of reaction at the tip increased still further with coelom formation. In still later stages the tips of the developing oral lobe and anal arms became the "high" ends of new ectodermal gradients. As the ciliated band developed, its cells also showed increase in reactivity above that of the general ectoderm. With gradual progress of starvation in the fully developed plutei the gradient differentials decreased, and before death almost completely disappeared. In these earlier data on oxidation gradient patterns it was not determined whether a differential in rate of oxidation was present between blastocoelar and outer surface of the cell-wall of the blastula and early gastrula, although it had been determined still earlier that dye reduction decreased in rate from the blastocoelar to the external surface (Child, 1936a, 1936b) and it had been suggested that this was probably due to lower oxygen content in the blastocoel than outside.

In a later, more extended study of indophenol reaction and reduction in normal *Patiria* development (Child, 1944) the rate of the polar indophenol oxidation gradient was found to decrease basipetally from the apical region in later oöcytes, cleavage stages, blastulae and early gastrulae. With progress of invagination a new oxidation gradient developed in the entoderm, as in *Dendraster,* with decrease in rate basipetally. A ventrodorsal oxidative gradient seemed to be visible in some late blastula stages, but is not indicated in the figures of these stages since it was difficult to determine when it first appeared. With further development it became more clearly visible and with coelom and stomodeum development other local oxidative patterns appeared. That paper, like the earlier studies of indicator patterns, in echinoderm development, was concerned primarily with polar pattern, and the indophenol reaction was allowed to continue until the polar and later local differentials became distinct, without much attention to the earlier stages of the reaction. However, it was indicated by the course of the arrows in certain figures, *e.g.*, Figures 11, 13–17, 19 and 20 (Child, 1944), that reduction in the cell-wall of blastulae and early gastrulae progressed from the blastocoelar surface outward. This differential was still regarded at that time as probably resulting from lower oxygen content in the blastocoel than outside and therefore of minor importance in development. It was not even determined whether a differential in indophenol reaction, an oxidative differential, appeared in the cell-wall in early stages of the reaction. As will appear in the present paper, an oxidase differential is present in the cell-wall of the blastula and early gastrula. The failure to observe it in this earlier investigation of indicator pattern is an interesting example of the influence which a preconceived opinion may have on observation.

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[2] It is perhaps unnecessary to note that "normal" development indicates merely the range of variations occurring under those conditions which we regard as natural. In its origin and determining factors it does not differ in any way from the experimental modifications of development under other conditions.

[3] The terms "apical" and "basal" are used for earlier developmental stages as less awkward than "animal" and "vegetal" or "vegetative" and as permitting use of the terms "basipetal" and "acropetal" in description of gradient patterns.

Redox indicator patterns of echinoderm exogastrulae have not been thoroughly studied. In the first attempt to learn something about indicator patterns of exogastrulae, only differential reduction of vital dyes was determined following staining by oxidized dyes with oxygen decrease in the external medium. These data are to be considered in a later paper. Patterns of intracellular oxidation of indicators have been determined only in *Dendraster* and only by means of the indophenol reaction and have been recorded only briefly (Child, 1941b). In that paper it was stated that in exogastrulae with enlarged and elongated entoderms indophenol reaction progressed from the entodermal tip toward the ectoderm. It was also noted that in the thick-walled blastulae which occur in exogastrulating agents and usually become exogastrulae, if not too much inhibited for further development, the cell-wall gradient progressed from the blastocoel outward, but the cell-wall oxidative gradient after actual evagination and elongation of the entoderm was not considered. In the less extreme types of exogastrulae in which the ectoderm approaches or attains pluteus form, the ectodermal oxidative pattern is essentially like that of the normal pluteus. In the more extreme forms of exogastrulae the ventrodorsal oxidative gradient is entirely absent. The polar gradient is present, at least in the evaginated entoderm, but may be completely absent in the ectoderm or that part of it which has not been entodermized. There again, it was not determined whether a cell-wall oxidative gradient was present in these elongated exogastrular entoderms.

In *Patiria* the indophenol reaction gradient, the oxidative gradient pattern, was determined only for normal development, and information concerning oxidative patterns of exogastrulae has been completely lacking up to the present.

OXIDATION PATTERNS OF NORMAL BLASTULAE, EARLY GASTRULAE AND EXOGASTRULAE OF DENDRASTER

The polar pattern of the indophenol reaction and of oxidation of vital dyes reduced by sodium hydrosulphite in the normal blastula is a distinct gradient decreasing basipetally from the apical region (Fig. 1, *A*), like those of other echinoderms, so far as known (Child, 1941b, 1944, 1953b). However, the most interesting feature of the blastula pattern and one which has been largely neglected, as already noted, is the cell-wall gradient, decreasing from the blastocoelar to the external surface. It becomes visible with oxidation of vital dyes as well as with the indophenol reaction. Since this is an oxidative gradient pattern it evidently cannot be due to lower oxygen content in the blastocoel than outside. The immigrating mesenchyme cells react essentially like adjoining cells of the cell wall. The same polar and cell-wall pattern are present in the basal region and in the invaginating entoderm of the early gastrula (Fig. 1, *B*), but the ectoderm soon becomes so thin that presence of a cell-wall gradient in it becomes uncertain, *i.e.*, the differential in the thin layer, if present, is not sufficient to be clearly visible, except in an apical thickening, as in Figure 1, *B*. In many individuals the cell-wall gradient still seems to be barely visible but it is not consistently distinguishable in all as development progresses. In general, the cell-wall gradient is most clearly visible in early stages of the indophenol reaction and dye oxidation. As intracellular concentration of indophenol or dye increases, it becomes progressively less distinct, and finally almost or quite indistinguishable.

Figure 1, *C* is a *Dendraster* exogastrula from a lot subjected to rather extreme change from low to high temperature. This and several other lots were kept after

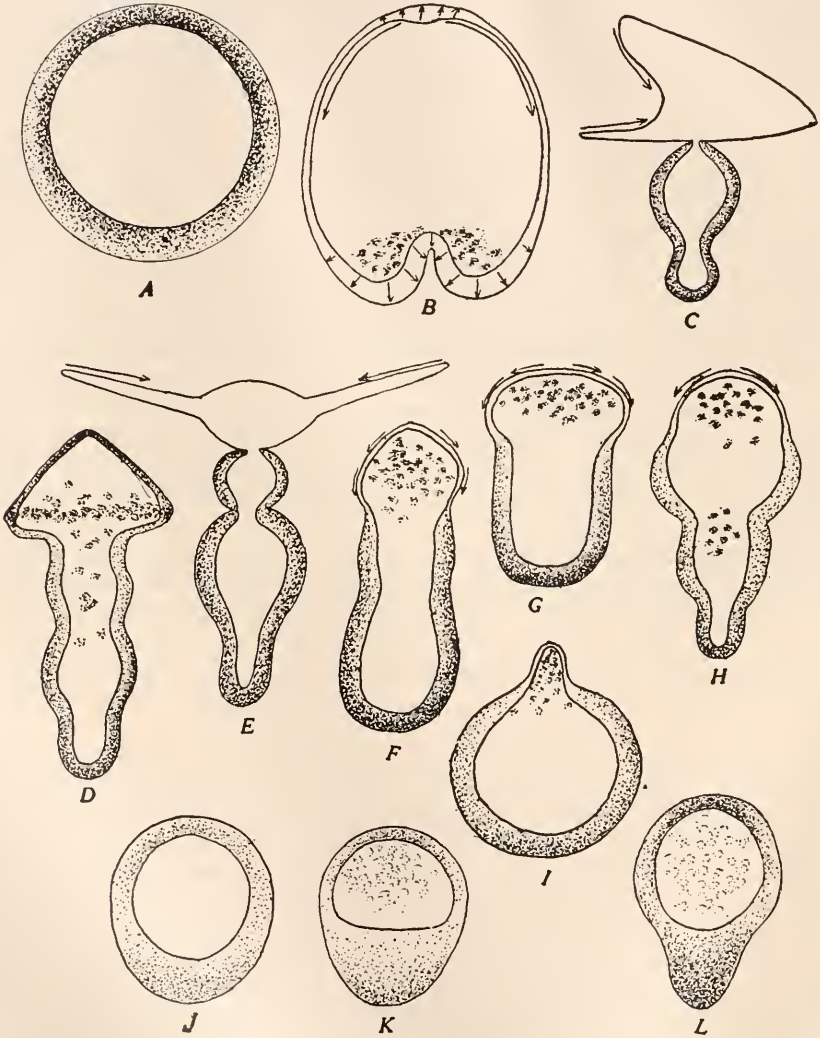

FIGURE 1. Oxidation patterns of *Dendraster excentricus: A,* normal blastula; *B,* normal early gastrula; *C-I,* LiCl exogastrulae; J-L, sodium azide pre-exogastrulae and early exogastrulae. For data concerning experimental conditions see the text.

fertilization at 8–10° C. and after 28 hours had developed to medium blastula stages. At this stage they were brought to a temperature of 20–22° C. for further development without any inhibiting agent. In the various lots this procedure resulted in exogastrulae ranging from an estimated 30 to 80–90 per cent, mostly of the less extreme types like Figure 1, *C*, with some degree of ventrodorsality and often approach to the pluteus form in the ectoderm. In the entoderm of Figure 1, *C* the polar gradient decreases from the tip toward the ectoderm and the cell-wall gradient from the external surface to the blastocoel, *i.e.*, opposite in direction to the cell-wall gradient in normal development.

Figures 1, *D–I* are various degrees of exogastrulation with exposure from the 4–8 cell stage to LiCl *M*/40 or *M*/50 for 24 hours or in Figure 1, *I*, 48 hours in *M*/50, followed by a day or two in water, at a temperature of 18–20° C. In *D*, as in *C*, the thin ectoderm is merely indicated in outline and gradient patterns by arrows. In the exogastrulae of *E-I* of Figure 1, the polar entodermal gradient decreases from the entodermal tip toward the ectoderm, and the cell-wall gradient from the external surface inward. This cell-wall pattern has been observed in many hundreds of *Dendraster* exogastrulae, the gradient of normal development, from the blastocoel outward, in none. In Figure 1, *E* the ectoderm is somewhat less inhibited than in *F-I* and its polar gradient is indicated by shading. In *I*, the ectoderm is almost absent and without distinguishable gradient.

Figures 1, *J*, *K*, and *L*, were in water for one or two days after 23 hours in sodium azide *M*/350 from the 8-cell stage at 20–22° C. throughout. In these, little or no differential recovery occurred after return to water. They are forms with thickened entodermal region, slight indication of evagination of entoderm and are pre-exogastrulae, or in *L* a slight degree of exogastrulation. In these, and large numbers of others in the same or lower azide concentrations, the polar entodermal gradient decreased from the tip toward the ectoderm, the cell-wall gradient from the external surface inward, as in LiCl exogastrulae, though usually with less differential than with LiCl. In these three examples of azide differential inhibition, the ectodermal cell-wall has remained relatively thick and the usual polar gradient was present in it, decreasing from the apical region, but with rather slight differential. A cell-wall gradient was also distinguishable in these ectoderms, decreasing from the blastocoel outward, *i.e.*, the same as in normal development. If reversal in direction of the entodermal cell-wall gradient is associated in any way with exogastrulation, reversal of the ectodermal cell-wall gradient is not to be expected when the course of its development is not altered. After a day or two in water entodermal dissociation, usually external, began in many individuals like Figures 1, *J–L* and the indophenol reaction was usually more rapid in the dissociated and apparently cytolyzed or cytolyzing cells than in intact entodermal cells.

The *Dendraster* blastula and early gastrula stages of Figures 1, *A* and *B* represent patterns observed in hundreds of control individuals. Their presence has been repeatedly confirmed by Dr. Olin Rulon. Also the entodermal patterns of Figures 1, *C–L* are selected cases only insofar as they are intended to show different degrees of exogastrulation and approaches to it. In all cases of the temperature, LiCl and azide forms, the entodermal cell-wall gradient is reversed in all degrees of evagination of entoderm and the approaches to it.

OXIDATIVE PATTERNS OF BLASTULAE AND EXOGASTRULAE OF STRONGYLOCENTROTUS PURPURATUS

Developmental stages of *S. purpuratus* are smaller than those of *Dendraster* and *Patiria* and the cell-walls become so thin in the course of development that this form is somewhat less favorable material than the others, particularly as regards the cell-wall gradient. Moreover, it has seemed from earlier use of indicators that gradient pattern in this form has somewhat less differential than in the other two echinoderms. The polar gradient and the ventrodorsal gradient, when not obliterated by inhibiting conditions, are distinct in developmental stages, but after the cell-walls have become thin it is sometimes difficult to determine with certainty whether a cell-wall gradient is present. The inhibiting agent used for exogastrulation also decreases gradient differentials, but after return to water and more or less differential recovery, they may again increase to some extent. As starvation of the larvae progresses, the differentials of oxidative gradient pattern decrease and before death usually almost disappear. However, in normal development and in exogastrulae before the cell-walls have become very thin the differentials of gradient pattern are similar to those of *Dendraster,* though apparently less "steep."

In Figure 2, *A* the polar and cell-wall gradients of indophenol reaction in the normal blastula are indicated. The oxidation pattern of dyes reduced by hydrosulphite is similar. Earlier stages of *S. purpuratus* were also examined in an attempt to determine at what stage the cell-wall gradient became distinguishable. Often repeated examination of the earlier cleavage stages indicated that this gradient, decreasing from the blastocoelar surface, became distinguishable soon after a distinct blastocoel appeared, *i.e.*, about at 32- or 64-cell stages or perhaps somewhat earlier. At these stages the differential is slight and becomes distinguishable only in the earlier stages of intracellular oxidation. It has often seemed to be more distinct in the basal regions of the embryos, perhaps only because the cells of that region are larger than others. Certainly in later stages there is less differential basally than in the apical region (Fig. 2, *A*).

Also, often-repeated attempts have been made to determine whether the same cell-wall gradient is still present during invagination of the entoderm in normal gastrulation, as it is, at least in early stages, in *Dendraster* (Fig. 1, *B*). As invagination progresses, however, the cell-wall becomes so thin that, although in many cases the cell-wall gradient, decreasing from the blastocoel outward, seemed to be present in early stages of intracellular oxidation, its presence was still regarded as questionable.

In Figure 2, *B,* after 20 hours in LiCl $M/20$ at 18–20° C. without return to water, there is a slight basipetal polar gradient and in the apparently beginning evagination of the basal region, the prospective entoderm, indophenol and dye oxidations decrease from the entodermal tip, *i.e.*, from the outer surface inward, a reversal of th normal pattern. Figure 2, *C* is a somewhat similar case, also after 20 hours in LiCl $M/20$ at 18–20° C. Here a double gradient is present in the entodermal region, on the blastocoelar side a slight gradient decreasing from the blastocoel for a short distance, and externally another slight gradient, decreasing from the tip of the entodermal region and from the external surface inward. In this individual complete reversal of the entodermal cell-wall gradient has not yet occurred. The mesenchyme cells oxidize about as rapidly as, perhaps slightly more rapidly than, the adjoining entoderm. The ectodermal polar gradient decreases basipetally and the cell-wall gradient remains as in normal development.

Figure 2, *D* represents an exogastrula two days in water after one day in LiCl *M*/40. In the thicker distal entoderm intracellular oxidation decreases from the entodermal tip and the cell-wall gradient from the outer surface inward. In the proximal entodermal segment a polar gradient is present with decrease toward the ectoderm, but the cell-wall has become so thin that presence of a cell-wall gradient is uncertain. In the ectoderm the polar and cell-wall gradients remain as in normal development.

The exogastrulae of Figures 2, *E* and 2, *F* were two days in water after 25 hours in LiCl *M*/50. Th entodermal polar gradient decreased from the tip, the cell-wall gradient from the external surface inward. In the case of Figure 2, *E* the ectoderm retained a slight polar gradient but no cell-wall gradient was distinguishable; in the exogastrula of Figure 2, *F* no ectodermal gradient was visible.

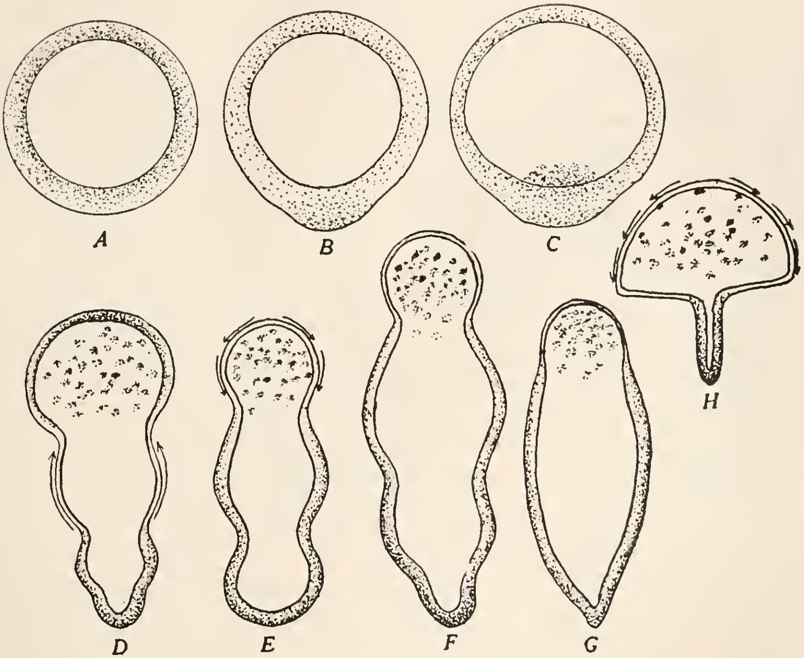

FIGURE 2. Oxidation patterns of *Strongylocentrotus purpuratus*: *A*, normal blastula; *B*, *C*, pre-exogastrulae; *D–H*, LiCl exogastrulae, some of mesenchyme cells becoming pigmented; data concerning experimental conditions in text.

The exogastrula of Figure 2, *G*, with the same treatment as *E* and *F*, was more inhibited than those, but with the same entodermal pattern and without distinguishable gradient pattern in the ectoderm. Figure 2, *H* from the same lot of eggs, with the same treatment and in the same container as *E–G*, is an example of the regional differences in inhibition which may occur in the same lot. The entoderm is more, the ectoderm less inhibited than in most animals of the lot, but the entodermal oxidation pattern is the same as in *E–G*. The ectoderm is thin, and only a polar gradient is distinguishable.

Similar oxidative patterns have appeared in material two days in water following two days in sodium azide $M/800$ and $M/1000$ at the same temperature range as the preceding cases. Since these forms presented nothing new, additional figures are regarded as unnecessary. Thus far no exogastrulae of *S. purpuratus* without reversal of the cell-wall oxidative gradient have been seen, though in later stages of exogastrular life the entodermal cell-wall often becomes so thin that presence of a cell-wall gradient becomes questionable. Also in pre-exogastrulae and cases of slight evagination double entodermal cell-wall oxidative gradients are not infrequently present, as in Figure 2, *C*.

Oxidative Patterns of Blastulae and Exogastrulae of Patiria

Of the three echinoderms included in this paper, *Patiria* is perhaps the most interesting as regards the cell-wall gradient and its alterations in relation to exogastrulation. The following data constitute the first evidence that an oxidation gradient, as well as a reduction gradient, is present in the cell-wall of *Patiria*. In the normal blastula and early gastrula the gradient pattern is like that of the echinoids. The polar oxidation gradient decreases basipetally from the apical region and at all levels the rate of indophenol reaction and dye oxidation decreases from the blastocoel outward in the cell-wall (Fig. 3, *A*). As the entoderm invaginates, a new gradient, decreasing from the entodermal tip, appears, as in the echinoids (Fig. 2, *B;* see also Child, 1944, Figs. 21–23). The entodermal cell-wall gradient persists, with decrease from the blastocoel outward; as the ectoderm becomes thin, the polar gradient is still visible, but in the thin cell-wall of the ectoderm of later stages it becomes impossible to determine whether a cell-wall gradient is still present.

The forms of Figures 3, *B–I* were in LiCl $M/50$ for 20 hours from late cleavage to early blastula stages. It was noted earlier (Child, 1953a) that these stages have been found more favorable for exogastrulation than exposure to the inhibiting agent in early cleavage. All developed at 18–20° C. The forms of Figures 3, *B* and *C* were a day in water after 20 hours in LiCl. In Figure 3, *B* the thickened, slightly evaginated entoderm shows an oxidation gradient decreasing from the outer basal surface and also a slight gradient decreasing from the blastocoelar surface of the entoderm. The ectodermal cell-wall gradient decreases from the blastocoel outward and the slight gradient on the blastocoelar side of the entoderm is merely persistence of a very slight differential of this gradient. In Figure 3, *C* the entodermal evagination has progressed much further and the cell-wall gradient decreases from the entodermal tip and from the external surface inward. In the thin ectoderm only a slight polar gradient is present as indicated. The exogastrulae of Figures 3, *D* and 3, *E* were two days in water after 20 hours in LiCl. In both, the characteristic entodermal exogastrular pattern and a slight polar ectodermal pattern appear. Figure 3, *E* is an example of the great entodermal elongation often appearing in exogastrulae of *Patiria*.

Exentogastrulae have been observed very generally in studies of echinoderm exogastrulation. They seem to occur more frequntly in *Patiria* than in the echinoids, but this may be merely a matter of degrees of differential inhibition and recovery or development of differential tolerance to the inhibiting agent. Figures 3, *F–I* are

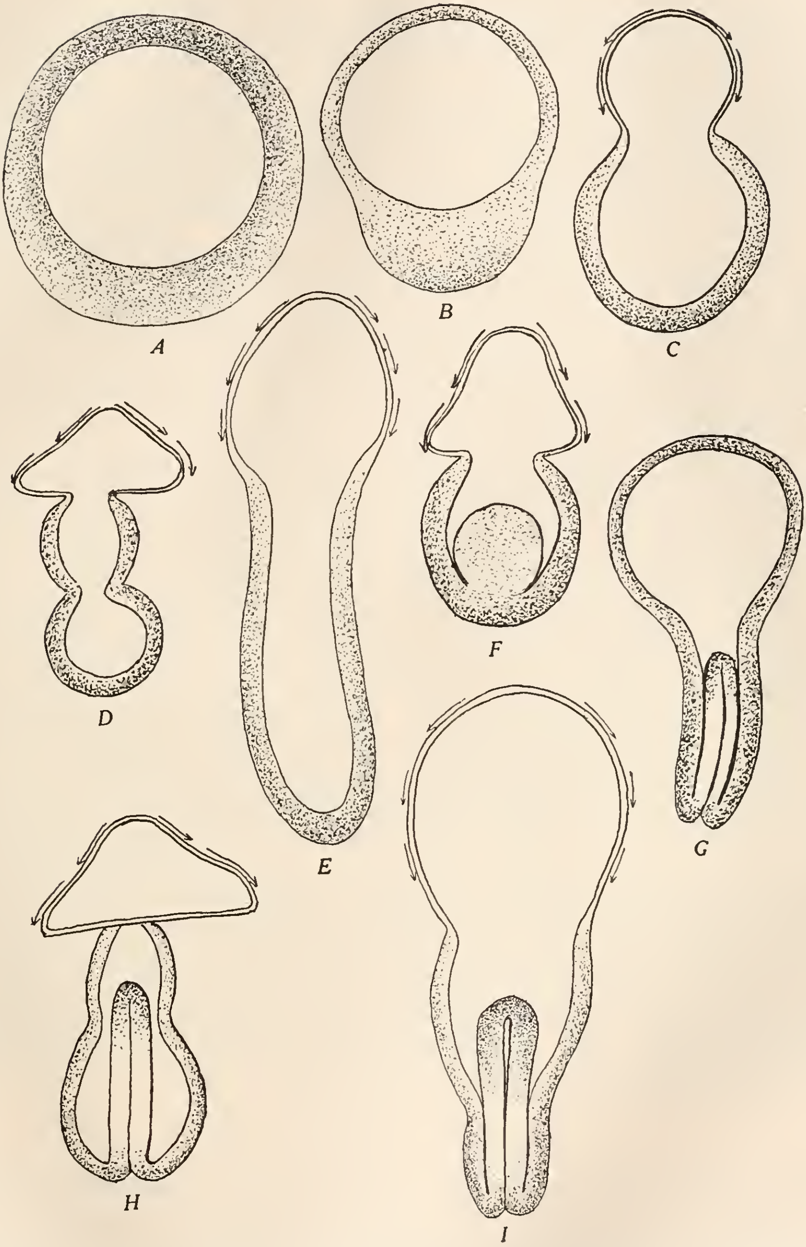

FIGURE 3. Oxidation patterns of *Patiria miniata: A,* normal blastula; *B-E,* different degrees of exogastrulation; *F-I,* exentogastrulae; all LiCl forms, further data in text.

examples of *Patiria* exentogastrulae in material 2–3 days in water after 20 hours in LiCl *M*/50. This concentration of LiCl and period of exposure did not prevent various degrees of differential recovery and an invagination of a part of the entoderm after return to water. In Figure 3, *F* the oxidation gradient pattern of the evaginated part of the entoderm is like that of other exogastrulae, a polar gradient decreasing from the tip of the evaginated region and a cell-wall gradient decreasing from the external surface inward. The invaginated part of the entoderm is a solid mass with a very slight gradient pattern. The most interesting feature of this pattern is that the slight differential present decreases from the tip of the invaginated mass and from its blastocoelar surface inward, at least in the part farthest invaginated. Figures 3, *G, H* and *I* are exentogastrulae with polar oxidation gradient decreasing from the tip, and the cell-wall gradient from the external surface of the evaginated part inward. In each of these exogastrulae the elongated invaginated part of the entoderm shows an oxidation gradient decreasing from its tip and in the cell-wall from the blastocoelar surface inward, like the cell-wall gradient of normal development. This gradient pattern did not extend over the entire length of the invaginated entoderm. In *G* and *I* this may have been due to the crowding of evaginated and invaginated parts of the entoderm and failure of indophenol agents to reach the more basal invaginated regions. However, in *H* there is no crowding of the two parts. It appears probable that this re-reversal of the cell-wall gradient pattern requires a certain length of time and that it takes place progressively from the tip along the invaginated part of the entoderm. The invagination and elongation indicate presence of a polar gradient in the invaginating region with its "high" end at the tip. It is also possible that the re-reversal may occur only at the higher polar gradient levels of the invaginated region; lower levels may not recover sufficiently to react in this way. Many years ago, and in numerous cases more recently, differential recovery after inhibition has been observed at higher gradient levels and persisting differential inhibition at lower gradient levels of the same echinoderm larva. In these exentogastrulae of Figures 3, *F–I* the ectoderm is thick enough only in *G* to show a cell-wall gradient decreasing from the blastocoel to the exterior, as well as a polar gradient, decreasing from the apical region. In the other figures only a slight polar gradient is distinguishable in the thin ectoderm.

Patiria, perhaps even more than the other available echinoderms, will undoubtedly repay further study of exogastrulation with different inhibiting agents. The data of the present paper on *Patiria* are by no means final, but, since they agree with the data on echinoids as regards the relation of the cell-wall oxidation gradient to exogastrulation, they are presented merely as part of the evidence that may perhaps throw some light on the physiology of exogastrulation.

Discussion and Conclusions

Earlier studies of redox indicator patterns in echinoderm eggs and early developmental stages have demonstrated the presence of a polar oxidation gradient decreasing basipetally from the apical region in normal development. Also a new oxidation gradient appears in the invaginating entoderm, with decrease from the entodermal tip (Child, 1941a, 1941b; 1944, 1953b). This paper gives further evidence for the existence of these patterns and demonstrates their presence in exogastrulae, except when the ectodermal pattern is completely obliterated by the inhibiting agent.

In the earliest use of indicators on echinoderm developmental stages, only intracellular reduction of vital dyes with external oxygen decrease was considered. With this procedure a reduction gradient through the cell-wall of blastulae and early gastrulae was observed (Child, 1936a, 1936b). It was assumed, without further evidence, that this cell-wall gradient pattern, decreasing from the blastocoel outward, was merely an incidental result of lower oxygen content in the blastocoel than outside, in consequence of oxygen uptake by the cells of the cell-wall or the parts of these cells adjoining the blastocoel, at a higher rate than diffusion of oxygen inward from outside.

Presence of a cell-wall oxidation gradient was of course not recognized in these studies of differential reduction. However, it is now evident that an oxidation gradient, as well as a reduction gradient, is present in the cell-wall of the normal blastula and early gastrula and probably later, and that both gradients decrease from the blastocoel outward. In view of the presence of this oxidation gradient decreasing from the blastocoel outward, there appears to be no adequate basis for the earlier suggestion that the cell-wall reduction gradient, also decreasing from the blastocoel outward, results from lower oxygen content in the blastocoel than outside. That suggestion was never more than a hypothesis to account for the cell-wall reduction gradient. Actually nothing is known concerning oxygen content in the blastocoel, as compared with outside, but it appears improbable that the cell-wall oxidation gradient decreases from a region of lower, to one of higher, oxygen content. The cell-wall oxidation gradient is a feature of normal development; the reduction gradient appears only after oxygen decrease, either by oxygen uptake of the individual sealed in a small volume of liquid, or by use of a reducing agent. Perhaps under these conditions oxygen content in the blastocoel may become lower than outside in consequence of greater oxidase activity adjoining the blastocoel. The two cell-wall indicator gradients, both decreasing from the blastocoel outward, can be demonstrated in the same individual, though of course not at the same time. The oxidation gradient appears under natural conditions, the reduction gradient only after oxygen decrease.

In the evaginated entoderm of the exogastrula the cell-wall oxidation gradient decreases from the external surface inward, *i.e.,* it is reversed, as compared with the normal individual. This reversal has been observed only in that part of the exogastrula which evaginates as entoderm. This may vary widely in extent in either direction from the normal region of prospective entoderm. It may include only the most basal part of this region, as in Figure 2, *H*. In the earliest study of gradient pattern in exogastrulae it was also shown that in *S. purpuratus* and *S. franciscanus*, with delay of exposure to LiCl to late blastula stages, only the most basal part of the prospective entodermal region evaginated and developed as entoderm, or evagination might not occur, and entoderm might not be clearly distinguishable from ectoderm. At this stage of echinoid development entodermal activity is increasing and it is more inhibited than ectoderm. Incidentally, it is a question of some interest whether at this stage inhibited prospective entoderm is ectodermized. Certainly all degrees of apparent ectodermization appear under these conditions (Child, 1936b, Figs. 36–44). Similar restriction of evagination occurs in *Dendraster* under similar conditions (Child, 1940, Figs. 74–76). From forms of this character, often with even smaller evaginated entoderms with reversals of oxidation gradients, at one extreme of exogastrulation, the region of evaginated entoderm and gradient reversal may extend in all degrees, not only over all of the prospective entodermal region, but also into the region of prospective ectoderm, until only a small knob of ectoderm remains in the apical region (Fig. 1, *I* above) or until the entire body is entodermized.[4] It is evident from the numerous studies on exogastrulation that, with exposure to the exogastrulating agent beginning in early developmental stages, the part of the individual undergoing evagination as entoderm in general extends farther apically as degree of effect of the agent increases. In a given lot of eggs, particularly if they are from different females, a wide range of sensitivity to exogastrulating action may occur. Consequently a wide range in the part of the animal undergoing evagination and reversal of the cell-wall oxidation gradient may occur in a single concentration of the agent. It is evident from these data that there is no regional difference in prospective ectoderm and prospective entoderm in early development which a single inhibiting agent cannot completely obliterate. It is also evident that in early stages lability increases basipetally, though perhaps not uniformly.

In normal pregastrular stages ectodermal development is more rapid and its gradient pattern soon becomes less labile than that of prospective entoderm, the apical region, the "high" end of the polar gradient, the least labile of all. It probably also becomes morphologically different from entoderm, as its further development suggests. With the gradual decrease in thickness of the ectoderm it becomes increasingly difficult to determine whether the cell-wall gradient persists in it. The basipetal differential in ectodermal lability apparently increases as the polar gradient differential increases from early stages onward.

It has seemed to be very generally true that the degree of inhibition necessary for entodermization of prospective ectoderm increases acropetally. It is necessary, however, to call attention here to certain forms of *S. purpuratus* appearing with inhibition by sodium azide, certain constituents of tobacco smoke and even with extreme crowding in water and in some cases with LiCl. In certain lots of material of this species all degrees of alteration of the apical ectodermal region from mere thickenings to outgrowths which are identical in appearance with evaginated entoderm at the basal pole of exogastrulae or in forms with invaginated basal entoderm appeared in large numbers in many containers. These apical outgrowths in many cases developed three segments, exactly like the basal entoderm (Child, 1948). In the first study of indicator patterns in exogastrulae somewhat smaller apical outgrowths, though often with three segments like basal entoderm, appeared very frequently with LiCl inhibition but were not included in published data, as it was desired to obtain further evidence as regards their occurrence. At present the only suggestion that seems to account for these forms is that the action of the agents was effective so early in development that alteration of the apical region occurred before its lability decreased appreciably. As the "high" end of the polar gradient it was more susceptible to inhibition and alteration than other levels of this gradient. If the agents were sufficiently effective very early in development, it seems possible that alteration and even entodermization of the apical region might occur without much effect on other levels of prospective ectoderm. *S. purpuratus* is in general more susceptible to inhibiting agents than *Dendraster* or *Patiria*. In *Dendraster* slight degrees of thickening and alteration of the apical region have been occasionally observed, but nothing that could be regarded as actual entodermization. Also only slight apical alterations have been observed in more recent *S. purpuratus* material. It is possible that the material of the 1948 paper was for some reason unusually susceptible or that the polar differential of early stages was greater than usual. Long experience with echinoderm material suggests that different lots may differ considerably in degree of reaction to external agents and in differential effects.

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[4] For earlier figures of this range of forms of exogastrulae see MacArthur, 1924, particularly Figure 2; Child, 1936b, Figures 1–5, 1940, various figures.

According to Figures 1, *J*, *K*, *L*, 2, *B*, *C*, *D*, and 3, *B* and *G*, above, there is no reversal in the cell-wall oxidation gradient as long as its ectodermal character persists. Apparently reversal occurs only when it is entodermized and takes part in evagination. The occurrence of reversal in the cell-wall oxidation gradient in evaginated entoderm raises what are perhaps the most interesting questions associated with exogastrulation. What determines reversal of this gradient? Does this reversal constitute a reversal of the physiological polarity of the entoderm cells or of the entodermal region in which it occurs? And finally, does the reversal determine reversal in the direction of entodermal growth, *i.e.*, evagination instead of invagination, or is it merely an incident or a result of an evagination determined in some unknown manner?

Considering first the question, how reversal occurs, it is evident that the cell-wall gradient represents a relatively slight differential. In a cell-wall only one cell thick it occurs between inner and outer ends of single cells. In solid entodermal masses it may involve multicellular regions. It is most clearly visible in the earlier stages of intracellular oxidation. As intracellular concentration of indophenol or oxidized dyes increases, it becomes difficult or impossible to distinguish it. In the presence of differentially inhibiting, exogastrulating agents this differential is undoubtedly decreased or perhaps completely obliterated with less inhibition than the polar pattern with much greater differential from apical to basal regions. The blastocoelar end of the cell-wall gradient, the region of higher oxidase activity, will undergo the greatest decrease. In various series it was observed in all three species that under inhibiting conditions the cell-wall gradient was very slight or could not be distinguished in many individuals which remained in blastula stages without definite invagination or evagination of entoderm.[5] As regards the intracellular conditions determining reversal of the cell-wall gradient in evaginating entoderm only suggestion is at present possible. Following decrease or obliteration of the original cell-wall gradient of evaginating entoderm, it appears highly probable that diffusion of oxygen inward, together with oxygen uptake of the cells, will establish a new gradient decreasing from the external surface. Less oxygen will reach those parts of the cells farthest from the external surface than those nearer the exterior.

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[5] More attention was given to this point in *S. purpuratus* material than in the other forms. In laboratory records concerning this species decrease or absence of the entodermal cell-wall gradient was noted in blastulae 20 hours in LiCl $M/20$, two days in $M/50$, two days in $M/60$, in a lot with extreme crowding in water and in a lot one day in water after one day in sodium azide $M/500$. In *Dendraster* almost complete absence of this gradient was noted after 20 hours in azide $M/600$, after 20 hours in LiCl $M/50$, and in blastulae still living after a day in water following 20 hours in azide $M/250$. In various other lots presence of this gradient was uncertain but this was not specially recorded. These are believed to be cases in which the original cell-wall gradient was in process of being obliterated by the exogastrulating agent.

In various other organisms, particularly among the hydroids, an oxygen differential from the free surface, perhaps also an opposed carbon dioxide differential from the surface in contact with the substrate are apparently the factors determining a new polar gradient pattern and various other modifications of morphological pattern (Child, 1941a, pp. 413–420; also pp. 425–6 and Fig. 144). Moreover, oxygen may be an important factor in the experimental determination of ventrodorsality in *Dendraster* (Pease, 1941, 1942a, 1942b). Echinoderm material exposed to exogastrulating agents is usually returned to water before actual evagination occurs. Except in cases of extreme inhibition, this permits more or less differential recovery and still greater opportunity for determination of a new cell-wall gradient. It appears possible, however, that even without return to water, diffusion of oxygen into the cell-wall, together with the oxygen uptake of the cells, though oxygen uptake is less than under natural conditions, may determine a new gradient, decreasing from the external surface inward.

As regards the question of reversal of entodermal polarity by the reversal of the cell-wall gradient, it is to be noted first that physiological axiate patterns of morphogenesis and gradient pattern have been shown to be closely associated in many organisms, both in embryonic development and in reconstitution in later life. When ventrodorsal gradient pattern is obliterated by inhibiting agents in early echinoderm development, completely radial forms develop, like most of the exogastrulae in the figures of this paper. When gradient patterns are almost or entirely obliterated by an external agent morphogenesis is almost or completely absent (*e.g.*, Child, 1948, Figs. 79–85).[6] In the case of echinoderm exogastrulation there seems at present to be no reason for doubting that reversal of the cell-wall gradient reverses, partially or completely, entodermal polarity.

In view of the very general association of gradient pattern and course and character of development it appears highly probable that this reversal of the cell-wall gradient and entodermal evagination instead of invagination are directly associated. The entoderm invaginates when it possesses a certain cell-wall gradient. When this pattern is reversed it is highly probable that it must evaginate.

In the case of exogastrulation resulting from exposure to a very low temperature in earlier stages with transfer later to a room temperature of about 20° C. (Fig. 1, *C* above), the low temperature during the earlier stages evidently acts like other exogastrulating agents and decreases or obliterates the cell-wall gradient. On transfer to the much higher temperature, the relation between the greatly increased oxygen uptake and the diffusion of oxygen inward probably determines the reversal. The exogastrulae produced in this way in *Dendraster* are almost entirely of the less extreme types, with ectoderm approaching or attaining pluteus differentiation, *i.e.*, ectodermal recovery is almost complete, but reversal of the entodermal cell-wall gradient and evagination occur.

One other point remains to be considered. With certain degrees of differential inhibition exentogastrulae appear more or less frequently, usually with the less extreme degrees of inhibition and after return to water and differential recovery. They seem to occur more frequently in *Patiria* than in the echinoids and in many of these forms the entire length of the entoderm becomes very great, as in Figures 3, *G, H,* and *I*. Moreover, in these cases there seems to be a re-reversal of the cell-wall gradient in the terminal region of the invaginated part. Except for this terminal region the invaginated part may be crowded inside of the evaginated part and absence of any gradient pattern in it may be due merely to failure of the redox agents to reach it (Figs. 3, *G* and *I*), though in Figure 3, *H* the cell-wall gradient is evident only in the terminal region, and in that case there is no crowding. All of the exentogastrulae of these figures and various others were returned to water after 20 hours in LiCl $M/50$ at room temperature.

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[6] For another case of obliteration of gradient pattern and of morphogenesis see Child, 1941a, pp. 167–69, Figure 57 and pp. 425–6 and Figure 144. On the other hand, new patterns with a variety of symmetries in addition to the polar pattern develop in the reconstitution of isolated pieces of *Corymorpha* in relation to the differential originating between the surface in contact with the glass of the container and the parts freely exposed to the water (Child, 1941a; pp. 413–420, Figs. 141 and 142).

The change from evagination to invagination of the entoderm is the feature of greatest interest in these forms. As regards conditions determining this change, it is suggested that the reversal of the cell-wall gradient may not be quite complete in the evaginated entodermal tip, that some enzymatic or other trace of the original gradient may persist but may be obscured or overlaid by the gradient decreasing from the external surface. With differential recovery after return to water the tip of the evaginated entoderm recovers most rapidly and most completely, and the original cell-wall gradient may be to some degree reactivated and become sufficiently effective to bring about the beginning of invagination in the entodermal tip. If the beginning of invagination is determined in this way increase in the differential of this re-reversal of the cell-wall gradient may be expected to occur, at least in the invaginating tip. In the blastocoel the relation between diffusion of oxygen from the blastocoel into the cell-wall and oxygen uptake of the cells may be regarded as the factor concerned in this increase. In the terminal regions of the invaginated entoderms of Figures 3, *G*, *H*, and *I* and even in the invaginated entodermal mass of Figure 3, *F* this pattern is distinguishable.

With increase in concentration of the exogastrulating agent or increase in length of exposure period exentogastrulae decrease in frequency and with sufficient inhibition do not appear at all, even after return to water. This relation to concentration of agent and exposure period seems to indicate complete obliteration or destruction of any basis or substrate for the original cell-wall gradient. The reversed gradient pattern has apparently become irreversible in these cases.

Figures 3, *F–I* show only later or final stages of exentogastrulae. It has not been possible thus far to identify the beginnings of exentogastrulation, *i.e.,* the pattern of the entoderm at the critical point of the change from evagination to invagination. The indicators injure or finally kill and it is not certain that particular individuals which may suggest a change in gradient pattern would go on to definite exentogastrulation if they remained alive.

In the entexogastrula with invagination followed by evagination, the inhibiting conditions are at first not sufficient to determine evagination of entoderm but become effective later. Their occurrence may be determined by delay of exposure to the agent to slightly later developmental stages than those ordinarily used for exogastrulation or by increasing concentration of the agent after an initial exposure. In these there is merely the single change from invagination to evagination of entoderm.

Further studies of *Patiria* exogastrulation in 1953 indicate that at least some of those forms with both evaginated and invaginated entoderms are entexogastrulae, rather than exentogastrulae. In those cases some degree of entodermal invagination is followed by evagination as effect of the inhibiting agent increases. Both invaginated and evaginated entoderm may continue to increase in length. In entexogastrulae the cell-wall gradient of the tip of the invaginated entoderm is not a "re-reversal" of pattern, but a persistence or a recovery of the original pattern, with decrease from the blastocoelar surface.

SUMMARY

1. A modification of the Nadi or indophenol reaction, using very low concentrations of agents, and intracellular oxidation of dyes reduced by sodium hydrosulphite, demonstrates the existence of a cell-wall oxidation gradient, decreasing from the blastocoel outward and from the apical region basipetally, in normal blastulae and early gastrulae of *Strongylocentrotus purpuratus, Dendraster excentricus* and *Patiria miniata.*

2. In the evaginated entoderm of exogastrulae, derived either from the original prospective entoderm or from entodermized ectoderm, the cell-wall gradient is reversed in direction, decreasing from the external surface toward the blastocoel.

3. The cell-wall oxidation gradient of ectoderm which persists as ectoderm does not undergo reversal, but as the cell-wall decreases in thickness, it becomes difficult or impossible to determine whether it persists.

4. Reversal of the cell-wall oxidation gradient by an exogastrulating agent is regarded as resulting from two factors: first, more or less complete obliteration of the original gradient by differential inhibition; second, from establishment of the reversed gradient by the relation between diffusion of oxygen inward from the external entodermal surface and the oxygen uptake of the entodermal cells.

5. It is suggested that this reversal of the oxidation gradient pattern reverses the polarity of the entoderm, and the reversed polarity determines evagination instead of invagination.

6. In exentogastrulae entodermal evagination is followed by invagination. In some of the exentogastrulae appearing in *Patiria* material a "re-reversal" of the cell-wall gradient occurs in the terminal region of the invaginated part of the entoderm. It is suggested that in the exentogastrulae some trace of the original cell-wall gradient persists, although the visible gradient is reversed. After differential recovery, following return to water, some degree of reactivation of the original gradient in the cell-wall of the entodermal tip, the region of most rapid and most complete recovery, determines the beginning of invagination, and conditions in the blastocoel bring about further reactivation in some individuals. Exentogastrulae decrease in frequency or do not appear at all with increasing degrees of inhibition, presumably because the original cell-wall gradient has been completely obliterated. In entexogastrulae there is no "re-reversal" of the cell-wall gradient but merely persistence or recovery of the original gradient.

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REGENERATION OF THE ANTERIOR END OF AULOPHORUS FURCATUS (NAIDIDAE) WITH SPECIAL REFERENCE TO EFFECT OF X-RAYS[1, 2]

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Aulophorus furcatus is an asexual, aquatic naid 3 to 7 mm. long and has the ability to regenerate very rapidly. The literature, however, reveals only taxonomic studies (Stephenson, 1930; Cernosvitov, 1944). Effects of irradiation on annelid regeneration have been almost entirely limited to total irradiation of varying doses (Stone, 1932, 1933; Turner, 1934, 1935). Zhinkin (1934) grafted lethally irradiated segments to normal hosts and attributed the resulting regeneration in the irradiated graft to a cell type, the neoblast, which had migrated from the healthy host. The neoblast is considered the vital factor by some investigators, while others believe it unimportant (Hämmerling, 1924).

In 1948, a technique of partially shielding an immobilized planarian exposed to x-radiation was reported (Wolff and Dubois, 1948). Their method was modified and applied to *A. furcatus*. Observations of the neoblasts in irradiated and non-irradiated worms were made in an effort to determine whether they contributed to regeneration in this species.

MATERIALS AND METHODS

Stock cultures of worms were maintained in glass dishes filled with distilled water and were fed boiled lettuce. Experimental worms were transferred to interlocking castor dishes. Amputations of the anterior ends were made at the sixth segment just posterior to the pharynx (Fig. 1). In the anteriorly irradiated worms, at least 8 and usually 12 to 15 segments were present between the wound and the shielded part of the worm.

The animals which had only the anterior half irradiated were immobilized by covering them with a thin layer of 2% agar. The agar containing the worms was cut into blocks. These were aligned on a watch glass and a three-mm. thick lead strip was placed over the posterior halves of the worms. They were then exposed to the determined lethal dosage of 2600 r at a rate of 300 r per minute. The radiation was produced by a 200 Kv air-cooled tube. A cardboard filter was used to minimize heat. Amputations of the head were made immediately after exposure.

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[1] This investigation was carried out under direction of Dr. H. W. Beams, in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Zoology, The State University of Iowa, Iowa City, Iowa.

[2] Irradiation by permission and under direction of Dr. T. C. Evans, Radiation Research Laboratory, The State University of Iowa, Iowa City, Iowa.

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Three series of worms were observed in detail: one non-irradiated, one totally irradiated, and one of anteriorly irradiated worms. Totally irradiated animals died 14 to 21 days after exposure to 2600 r. Normal regeneration was completed during the fifth day. At selected intervals following amputation, the worms were fixed in Bouin's solution. Selected sagittal sections were stained with Delafield's hematoxylin and eosin Y and microphotographed at 600 diameters.

No grafting experiments were made. In preliminary work, it was observed that this species will discard any injured segment. Healing immediately follows and no fusion would occur. The fixative was also the killing agent as this worm rapidly autolyzes immediately following death. General observations were made on regeneration of the tail of normal and posteriorly irradiated animals.

RESULTS

The observations on the non-irradiated worms indicated a sequence of recovery similar to that described in other species of the Naididae (Krecker, 1923; Stephenson, 1930). The initial response was an epidermal healing with no mitoses evident (Fig. 4). Simultaneously, the severed end of the alimentary tract closed over. In three to six hours, a migration of the neoblasts was evident (Fig. 3). Next, the epidermal scar thickened and some cell division occurred, after arrival of neoblasts. This thickening proceeded until the epidermal cap was formed, the newly formed cells seemingly pushed inward. These, together with the proliferating endodermal cells of the intestine, formed a column or cord of cells termed the cell strand. Between the dorsal side of the cell strand and the epidermal cap, a cluster of epidermal cells formed a primordial cerebral ganglion which was well developed after 12 hours.

Continued canalization of the cell strand formed the regenerated anterior alimentary tract. The muscular layers of the intestine developed into the thicker walls of the new pharynx. The fate of the neoblasts was not positively determined. That some neoblasts enter into the formation of mesodermal tissues is likely considering the numbers present before cell differentiations had begun. The neural structures apparently developed from existing ganglion cells and the cells of the regenerated cerebral ganglion. An average of five days passed before the regenerated head was used in feeding and locomotion. The appearance of a normal and regenerated head is shown in Figure 1.

In the totally irradiated specimens, no regeneration occurred. An epidermal healing succeeded amputation in two to four hours. The scar thickened more than in non-irradiated worms. Neoblastic activity was never evident. The size of the existing cells became progressively smaller until death resulted (Fig. 2).

The anteriorly irradiated worms had an immediate healing response and neoblasts in the shielded segments were active four to six hours after amputation. There was a delay in the initiation of regeneration of 12 to 24 hours (Figs. 5 and 6). Other than this, regeneration proceeded as in the non-irradiated worms. Some of the irradiated worms shed the cuticle from the exposed segments on the second or third day. Complete morphological recovery required one to two additional days. Functional recovery necessitated two to four days longer than in the non-irradiated worms.

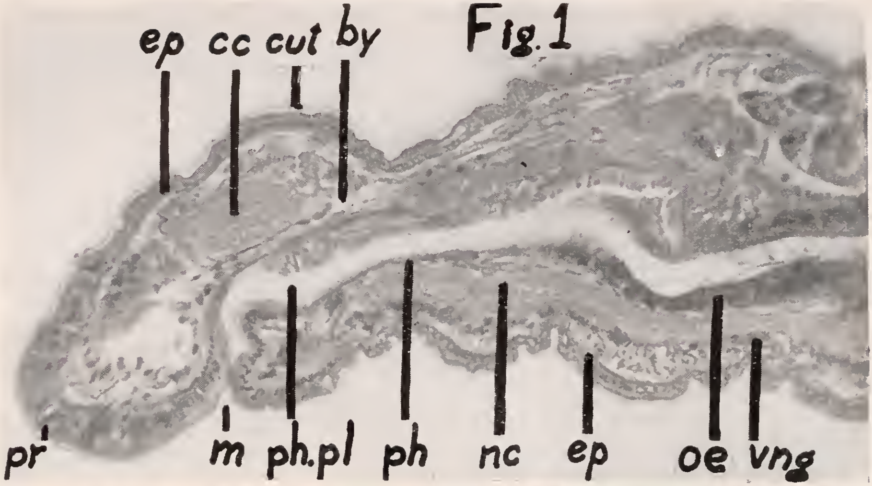

Fig. 1

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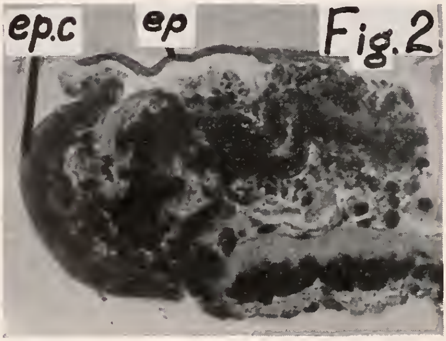

Fig. 2

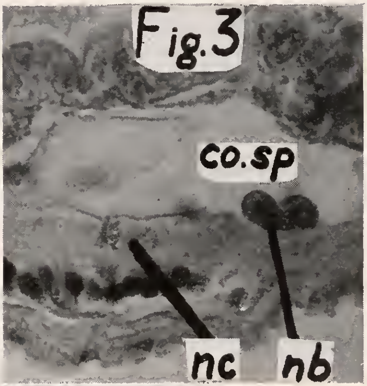

Fig. 3

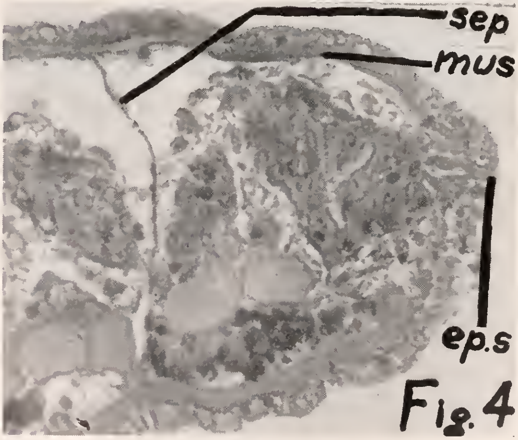

Fig. 4

Figures 1–4.

In posterior regeneration, in both irradiated and non-irradiated worms, the neoblasts were seen migrating to the wound site. No detailed microscopy was made of posterior end regeneration.

DISCUSSION

The results indicated that the neoblasts, in this species, were involved in the regeneration process, either directly or indirectly. The presence of the neoblasts in the wound area contributed, in some way, to initiation of mitotic activity in the epidermal cap and cell strand. It is probable that the neoblasts form some of the regenerated mesodermal tissues. Several workers, using many neoblastic species, have concluded that the neoblast is essential to regeneration (von Wagner, 1906; Krecker, 1923; Sayles, 1927; Zhinkin, 1934). Contrary arguments as to the importance of neoblasts have been presented by other workers (Hepke, 1897; Abel, 1902). They attributed no function to the neoblast in the regeneration process. In all of these experiments, however, irradiation was not used.

Irradiation experimentation on two annelid species gives support to the contention that neoblasts, when present, do have an important role in regeneration. Irradiation of the neoblastic species, *Tubifex tubifex* (Stone, 1932, 1933) and *Lumbriculus inconstans* (Turner, 1934, 1935), revealed that the regenerative processes were halted or delayed. The conclusion was that the embryonic neoblasts were killed or severely inhibited. Similar experiments and results on species of planaria have been reported (Curtis, 1936). In *Rhynchelmis limosella*, irradiated segments were grafted to a non-irradiated host (Zhinkin, 1934). Regeneration in the graft began after the neoblasts had migrated from the host through the graft to the wound area. Observations on a planarian species in which one-half of the animal was irradiated have been reported (Wolff and Dubois, 1948). It was noted that regeneration in the distal portion of the irradiated half occurred after neoblasts had migrated from the non-irradiated portion to the wound area. No neoblasts were

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FIGURES 1–4.

Abbreviations are as follows:

bv.	blood vessel	m.	mouth
cc.	cerebral commissure	mus.	muscle layer of body wall
cg.	cerebral ganglion	nb.	neoblast
co. sp.	coelomic space	nc.	nerve cord
cs.	cell strand	ph. pl.	pharyngeal plate
cut.	cuticle	ph.	pharynx
ep. s.	epidermal scar	pr.	prostomium
ep. c.	epidermal cap	vng.	ventral nerve ganglion
ep.	epidermis	sep.	septum

All sections are sagittal.
Magnification is 600 × except Figure 3 which is 900 ×.

FIGURE 1. Mid-sagittal section of head of normal worm.

FIGURE 2. Anterior end of specimen 18 days following total irradiation of 2600 r. Head was amputated immediately following exposure. No cell strand. Reduced body diameter, all structures atrophied.

FIGURE 3. Two neoblasts migrating anteriorly along sheath of nerve cord. Large nuclei, prominent nucleolus. Section made of specimen six hours after amputation of head.

FIGURE 4. Four hours after amputation of head showing epidermal healing.

evident in the irradiated half. The most extreme interpretation of the regeneration role of the neoblasts was that of Hämmerling (1924). He compared the neoblasts to the meristem cells of plants or the interstitial cells of coelenterates.

The migratory ability of the neoblasts in *A. furcatus* was more extensive than that reported in other species. Migration toward the posterior end only was re-

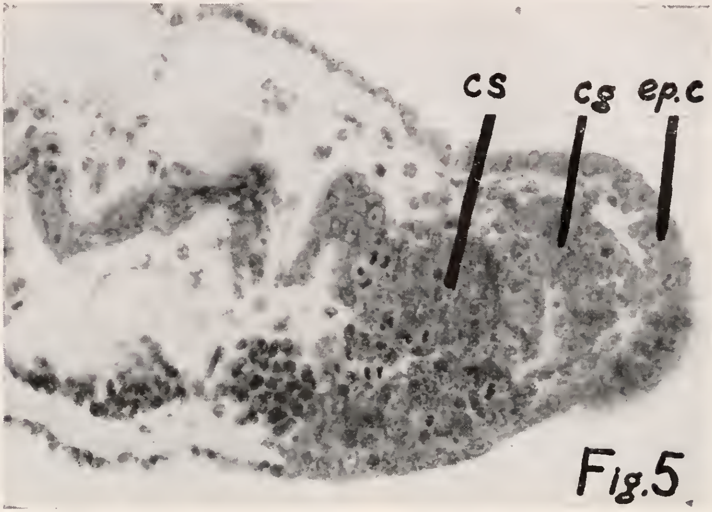

Fig.5

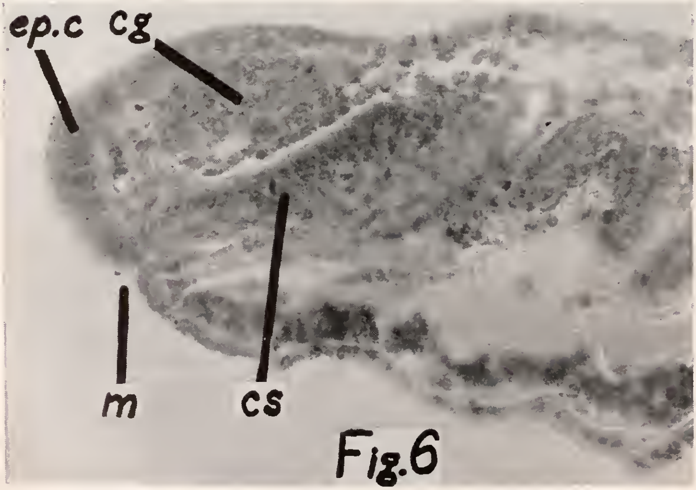

Fig.6

FIGURE 5. Forty-eight hours after head amputation of anteriorly irradiated worm. Many mitoses present. Mouth notch not yet present. Cell strand not as definite as in Figure 4.

FIGURE 6. Forty-eight hours after head amputation of normal worm. Cell strand well developed, mouth notch present. Cerebral ganglion well developed. Mitoses infrequent. Prostomium taking form.

ported in *Lumbriculus* (Sayles, 1927) and a maximum anterior migration of 7 to 9 segments in *Limnodrilus* and *Tubifex* (Krecker, 1923). In *A. furcatus,* anterior migration was observed through 15 or more segments.

The delay in the initiation of regeneration can be explained only in part by the time taken by the migration of the neoblasts. The general effects on other tissues (Clark, 1940; Lea, 1947) also contributed to the delay. Replacement of some cells and recovery from irradiation injury by the tissues, particularly the epidermis and endoderm, were necessary.

The posterior end of the worm is considerably different from that of the majority of oligochaetes. It has three pairs of gill-like processes and a pair of palps extending beyond the anal pore. Details of the histology of this posterior structure are yet to be recorded. Hence, posterior regeneration merited only general observation.

Summary

1. A comparative study of regeneration of the head of *Aulophorus furcatus* in non-irradiated, anteriorly irradiated and totally irradiated worms has been presented. Totally irradiated worms failed to regenerate a new head. A delay in the initiation of regeneration and rate of regeneration was noted in the worms which had the anterior half exposed to lethal irradiation. The lethal irradiation apparently had its effect by killing or severely inhibiting the neoblasts.

2. In this species, it was demonstrated that the neoblasts are capable of migrating both anteriorly and posteriorly. They are able to migrate into irradiated areas. The neoblast apparently contributes, directly or indirectly, to the regeneration process of both non-irradiated and irradiated specimens. The neoblast probably forms some of the new mesodermal tissues.

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A STUDY OF THE MECHANISM OF ACTIVATION AND NUCLEAR BREAKDOWN IN THE CHAETOPTERUS EGG[1]

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The phenomenon of stimulation and response has been studied in a wide range of biological systems by a considerable number of investigators. Because of the many advantages of marine egg material, numerous workers have devoted their efforts to an examination of stimulation or activation[3] as it occurs in this material. These workers have believed that the phenomenon of stimulation and response is based on a fundamental cellular mechanism, the response to which, in eggs, is manifested by the initiation of some aspect of development. Some have limited their investigations to the maturation process, or more strictly the breakdown of the germinal vesicle nucleus, since they reasoned that this response was not complicated by as many factors as is cleavage.

The investigations reported here were concerned with nuclear breakdown in the immature eggs of the annelid *Chaetopterus pergamentaceus*. This material is especially favorable for study because of a number of characteristics not present in other available forms. For one thing, 100% of the eggs will show nuclear breakdown in a large majority of the worms, thereby insuring rather good reproducibility of results. Furthermore, nuclear breakdown occurs spontaneously in sea water, eliminating the necessity of artificial activation. These factors were in addition to the advantages existing also in other material—such as an easily detectable response and a short time period between stimulation and response.

Until 1950 only one paper, that of Allyn (1912), had appeared in which an attempt was made to explain, by experimental procedures, nuclear breakdown in Chaetopterus eggs. However, some work has been done on eggs of other forms. This earlier research on the process of nuclear breakdown includes the investigation of the effects of pH and ions on Pomatocerus eggs by Hörstadius (1923); a considerable amount of work, mostly concerned with effects of salts, by Dalcq (1928) and Pasteels (1935) on a variety of forms; experiments by Heilbrunn and Wilbur (1937) on the role of calcium ions in the initiation of nuclear breakdown in Nereis eggs; some work by Scheer and Scheer (1947) on the effects of drugs and ions in the activation of Urechis eggs; and some investigations by LeFevre (1945) concerning the effects of picric acid on the activation of Nereis eggs. There exists, of course, a much larger literature on fertilization and artificial parthenogenesis, phenomena which are related—in terms of activation—to the process of nuclear breakdown in oöcytes. The above mentioned papers, as well as reviews by Lillie (1941), Tyler (1941), Runnström (1949), Danielli (1951), and Heilbrunn (1952), may be consulted for a more extensive bibliography on the various aspects of activation in general.

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[1] This paper represents a part of a dissertation in Zoology presented to the faculty of the Graduate School of the University of Pennsylvania in partial fulfillment of the requirements for the degree of Doctor of Philosophy. Part of this investigation was supported by a research grant from the National Cancer Institute, National Institutes of Health, Public Health Service, administered by Dr. L. V. Heilbrunn.

[2] Predoctoral fellow of the National Cancer Institute, U. S. Public Health Service, 1952–53. Part of the work was performed during the tenure of this fellowship.

[3] In order to avoid any possible confusion with specific embryological terms, activation is defined here as any response to stimulation (either in egg cells or in other types of living cells). In this instance, it is the initiation of the cellular activity leading to nuclear breakdown.

So long as the eggs of Chaetopterus are retained within the parapodia of the worm their large (germinal vesicle) nuclei remain intact. This is apparent from the fact that if the eggs are examined within the first 5 minutes after they are shed, the large nucleus with its membrane can be seen in 100% of the eggs. Within 7 or 8 minutes after the eggs reach sea water, the nuclear membrane disintegrates and some of the nuclear material migrates to the periphery of the egg where the metaphase spindle of the first maturation division is formed. It has been the aim of this research (some of which is reported here) to answer, in physical and chemical terms, three questions relating to the phenomenon of nuclear breakdown as it occurs in the immature Chaetopterus egg.

Since the stimulus for the breakdown of the nucleus occurs as a result of the release of the egg from the animal into sea water, the first question posed was—is the initial activation a result of a direct external stimulus or is it a result of a release from an inhibition? As noted earlier in a preliminary note (Goldstein, 1950), the activation is apparently produced by the release of the egg from an inhibitory environment. This conclusion was based on the observations that nuclear breakdown would occur in the presence of any one of the four common cations (Na, K, Ca, and Mg), in the presence of any of several anions (Cl, NO_3, HCO_3, and Br), in sucrose solutions, in hypertonic and hypotonic sea water, at any pH between 3.5 and 9.0, and in the absence of oxygen. Thus, it was concluded that no factor in the sea water is responsible for the stimulus, but rather that activation results from a release from an inhibition. This view agrees with the opinion of Allyn (1912) and the results obtained by Scott and LeBaron (1950). The foregoing information led to the formulation of the second question, namely, what is the nature of the inhibitor present in the ovarian environment? Though a substantial amount of work has been done, most of the data concerning the inhibitor have been negative. At this time it is possible to say with assurance only that the inhibitor is heat labile and of a small enough molecular size to be dialyzable. (In a previous report (1950) it was stated that CO_2 might possibly be at least partially responsible for the inhibition. More recent and more extensive examination has indicated that this position is no longer tenable.) The third question is the one with which this paper is primarily concerned: What is the intracellular mechanism that produces the nuclear breakdown?

As a result of some early experiments, which revealed that calcium played an essential role in the activation process (*cf.* Goldstein, 1950), I was prompted to employ the colloid chemical theory of stimulation of Heilbrunn (1952) as a working basis for experimental design. This theory states, in part, that *any* stimulus applied to the cell will release calcium ions from a bound state, and that the calcium ions will then react with the protoplasmic colloids to produce the specific cellular response. This view holds, moreover, that the mechanism is analogous to that involved in the clotting of blood. The approach employed here was that of "dissecting" the mechanism in such a manner that each individual step might be examined separately, following—to some extent—the approach used in interpreting the steps in blood clotting reactions.

I am grateful to Dr. L. V. Heilbrunn for suggesting this problem and for his willing encouragement, help and interest during the course of the work.

Material and Methods

Male and female Chaetopterus worms, obtained from the Woods Hole, Mass., area, were kept in separate fingerbowls under running sea water. Worms kept in this fashion did not often shed gametes in the laboratory and could frequently be maintained in good condition for as long as two or three weeks. Eggs and sperm were obtained by cutting as many posterior parapodia from the worm as would supply sufficient material for a particular experiment, and then the parapodia were cut open (into the appropriate solution) to release the gametes. In those instances where the eggs were shed directly into solutions other than normal sea water, the worms were first rinsed with distilled water and dried externally with filter paper in order to prevent contamination with substances in the sea water. In order to be able to carry out certain experimental procedures within two minutes after the eggs were shed, some preliminary screening was carried out. As a result, a few deviations from the commonly accepted handling of Chaetopterus eggs were employed. Thus, it was determined that straining and washing of the eggs were ordinarily unnecessary for normal nuclear breakdown, once the parapodia were cut open in sea water. (Usually between 10 and 20 parapodia were cut into a dish containing 15–20 ml. of solution.) The processes under study were apparently unaffected by any extraneous material from the worm. Furthermore, it has also been observed that virtually no degree of crowding of the eggs would affect the progress of nuclear breakdown.

Two criteria for determining normality of the eggs were used. First, the eggs shed into sea water were expected to yield nuclear breakdown in 100 of 100 eggs. This expectation was realized in 90–98% of the worms. The second criterion, generally observed, was that nuclear breakdown would occur within 7 or 8 minutes after the eggs were placed in sea water. Counts were made of at least 100 eggs from a dish, except in experiments where it was necessary to make rapid counts, in which case only 50 eggs were counted. In all the work reported here the data were of such a nature as to warrant no further statistical treatment. To examine and count the eggs it was necessary to remove a drop of egg suspension to a slide and flatten the eggs with a coverslip. This was done in order to determine the presence or absence of the nucleus (but even without flattening it was often possible to see the clear polar region of the first maturation metaphase spindle).

In many experiments, though not in all, the normalcy of the eggs before and after treatment was determined by fertilizing the eggs upon their return to normal sea water. Fertilization (and subsequent cleavage) was taken as an indication of viability in a sufficient proportion of the experiments in a series to reveal whether or not a particular experimental technique involved irreversible damage. Experiments with various types of inhibitors were considered valid only if the inhibition were reversible.

In utilizing CO_2 the following procedure was employed. The gas was bubbled into the solution through fine bolting cloth covering the opening of a glass tube leading from the tank. Since the gas was bubbled through under relatively low pressures, it was not possible (with the equipment available) to determine accurately the quantities of gas being delivered, and only rough estimates were made. Indeed, more exact estimates were hardly necessary and, in view of the fact that the material behaved in consistent fashion, the conclusions drawn from the experiments are believed to be valid.

Measurements of pH were all made with a Beckman pH meter.

In using the hand centrifuge for determinations of the rigidity of the cell cortex, the technique described by Wilson (1951) was employed.

Sea water modifications were made up according to Table I and were adapted, with certain alterations, from the analyses provided by Wattenberg (1938).

TABLE I

Sea water modifications

	NaCl	KCl	$CaCl_2$	$MgCl_2$	$MgSO_4$	$NaHCO_3$
Artificial sea water	410	8.85	10.2	20.7	29.6	2.0
Ca-free sea water	428	9.3	——	20.7	29.6	2.0
Ca- and Mg-free sea water	454	10.1	——	5.7 (K_2SO_4)	24.1 (Na_2SO_4)	2.0
Mg-free sea water	426	9.7	10.2	5.7 (K_2SO_4)	24.1 (Na_2SO_4)	2.0

Figures represent ml. of molar stock solutions of the various salts. In each case, distilled water is added to make the final volume one liter.

The sodium citrate solution used throughout the investigation consisted of: 50 parts of 0.35 *M* sodium citrate plus 50 parts of Ca-free sea water. This solution will hereafter be called the *citrate solution.*

In analyzing for proteolytic activity, the method of Anson (1938) for trypsin determination with a hemoglobin substrate was followed as closely as possible. Incubation was carried out for one hour at 25° C. and the Folin-Ciocalteau reagent was added to color the reaction products.

RESULTS AND DISCUSSION

Cortical changes

Wilson (1951) has described physical changes in the protoplasmic cortex of Chaetopterus eggs during the mitotic cycle and these have been related to Heilbrunn's theory of stimulation and response. If the theory is correct, we should expect that somewhat similar changes would occur if the eggs were activated to produce nuclear breakdown. For reasons that will become evident shortly, a study was made of cortical changes in the maturing egg only during the first few minutes following activation (see Wilson for method).

Eggs were shed into artificial Ca-free sea water, which was somewhat inhibitory to nuclear breakdown.[4] *Two* minutes after the eggs were placed in the Ca-free sea water they were centrifuged at various rates for one minute, removed from the centrifuge tube, placed on a slide with a coverslip and examined for the presence of cortical granules. The absence of granules in the cortical region of the cell is taken as an indication that the cortex had become less rigid and could no longer resist the centrifugal forces applied.

Table II shows that the percentage of eggs in which the cortical granules were moved by centrifugation was always in agreement with the percentage of eggs showing nuclear breakdown as observed at 8 minutes after the eggs were shed. (The observations on the cortex were made at about 4 minutes after shedding—before the nuclei have broken down.) Further evidence was offered by the fact that if the eggs were centrifuged following nuclear breakdown, the eggs which retained intact nuclei always had a more rigid cortex. This alone, however, could not be considered seriously as proof of a correlation between cortical change and nuclear breakdown since it was impossible to control for the presence or absence of the nucleus and its effect on cortical rigidity. Therefore, the experiments described in Table II—in which all the eggs had intact nuclei at 2–4 minutes—were regarded as significant and indicated that those eggs in which the nucleus was destined to break down had a less rigid cortex.

TABLE II

Nuclear breakdown and cortical rigidity

% eggs with cortical granules moved at 2–3 minutes	% eggs showing nuclear breakdown at 8 minutes	Centrifugal force in gravities
28	25	4150
42	40	9340
10	9	2335
10	11	2335

A liquefaction of the protoplasmic cortex following the fertilization of Chaetopterus eggs has been reported by Wilson (1951). Moser (1939) has also described cortical changes following stimulation of Arbacia eggs. Wilson and Heilbrunn (1952) have interpreted these cortical changes to be a reflection of the release of calcium ions from binding to the cortical proteins and this, of course, would alter the consistency of the protoplasmic gel there. In this study it appears that the liquefaction of the cortex occurs as a necessary prelude to nuclear breakdown and the next sections will show what role calcium plays in the process.

Role of calcium

That calcium plays a significant role in the activation of eggs has been shown by a considerable amount of work. Perhaps the most important was the contribution of Heilbrunn and Wilbur (1937). They demonstrated that the artificial induction of nuclear breakdown in Nereis eggs by ultraviolet light, NaCl or KCl was dependent on the presence of ionic calcium in the cell. If the eggs were placed in sodium citrate solutions, nuclear breakdown would not occur, citrate presumably making calcium unavailable for cellular activity. Repetition and extension of this work with Chaetopterus eggs was felt to be in order, since with this material no artificial measures are necessary for activation and, possibly, a different type of mechanism is at work.

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[4] This solution was made up as follows: 94 gms. NaCl, 20 gms. $MgCl_2$, 15.7 gms. Na_2SO_4, 2.7 gms. KCl, 0.77 gm. $NaHCO_3$, 0.1 gm. H_3BO_4, and 3983 gms. H_2O. Eggs shed into this solution produced only about 10–40% nuclear breakdown. These intermediate numbers (rather than 0% or 100%) permitted a basis for comparing the frequency of cortical change with the frequency of nuclear breakdown. The inhibition was reversible, and so the solution proved to be a useful tool in this instance.

Eggs were shed directly into a citrate solution (see Methods). This solution inhibited nuclear breakdown in ca. 90% of the eggs. If, 6 or 7 minutes after shedding into citrate, some of the eggs were placed in normal sea water and some were placed in Ca- and Mg-free sea water (see Table I), nuclear breakdown occurred in 100% of the eggs in normal sea water while a substantial percentage of those eggs in Ca- and Mg-free sea water was inhibited (see Table III).

These results suggested, of course, that divalent cations were necessary within the egg for activation to occur. When, however, the experiment was repeated, this time placing the eggs (from citrate) either into normal sea water or Ca-free sea water (Mg present), it was observed that again all the eggs in normal sea water produced nuclear breakdown, while there was a high percentage of inhibition of those eggs in Ca-free sea water as seen in Table IV. Apparently Mg is not the necessary cation since it is supplied in the Ca-free sea water. Therefore, it would appear that Ca is the important factor.

TABLE III

Experiments indicating a requirement for divalent cations (Ca and Mg)

Eggs shed directly into normal sea water	Eggs shed directly into Ca- and Mg-free sea water	Eggs shed directly into citrate and then, at 6–7 minutes, transferred to:	
		Normal s. w. or	Ca- and Mg-free s. w.
100	100	100	40
100	96	100	18
100	99	100	7

(Figures indicate % eggs showing nuclear breakdown.)

A third series of experiments (Table V), in which Mg-free sea water (containing Ca) was used instead of Ca-free sea water, proved to be conclusive. The results of these experiments showed no difference in the percentage of nuclear breakdown of the eggs when they were removed from citrate to either normal sea water or Mg-free sea water. Indeed, nuclear breakdown occurred in 100% of the citrated eggs in both Mg-free sea water and normal sea water. This experiment confirms the view that Ca and not Mg is important for the mechanism responsible for the dissolution of the nucleus.

The results of these three series of experiments (as well as a considerable amount of unpublished work) amply demonstrate the need for Ca in the mechanism of nuclear breakdown in the cell, and are in agreement with the work of Heilbrunn and Wilbur (1937). (The role of Ca in the activation of cells is discussed by Heibrunn,

Table IV

Experiments indicating a requirement for calcium ions

Eggs shed directly into normal sea water	Eggs shed directly into Ca-free sea water	Eggs shed directly into citrate and then, at 6–7 minutes, transferred to: Normal s. w.	or Ca-free s. w.
99	58*	100	6
100	52*	100	7
100	81*	100	24

(Figures indicate % eggs showing nuclear breakdown.)

* The controls in Ca-free sea water (without prior citrate treatment) were also inhibited (nuclear breakdown: 52–81%), but not to the extent that the eggs treated with citrate were (nuclear breakdown: 6–24%). It was shown (unpublished experiments done in another connection) by more conclusive means that, if 0.53 *M* NaCl were used instead of Ca-free sea water (right hand column of Table IV) calcium was again necessary for nuclear breakdown in eggs pretreated with citrate—whereas the controls in NaCl (without prior citrate treatment) showed 100% activation. Apparently the inhibitory effect of Ca-free sea water (without prior citrate treatment) here was due to some type of ion antagonism.

and his book (1952) should be consulted for a review of his theory.) Lindahl (1937) has questioned the results of Heilbrunn and Wilbur, claiming that the inhibition by citrate could very well be due to acidity. However, the work reported here shows that, following citrate treatment, the eggs may be placed in almost any environment lacking Ca and still be inhibited. It is only when Ca is returned to the cell that activation occurs and, consequently, the inhibition can hardly be attributable to a pH effect.

Sequence of steps in the activation mechanism

In view of the fact that CO_2 (Goldstein, 1950) and sodium citrate both reversibly inhibit nuclear breakdown, it was decided to investigate the possibility of a stepwise mechanism in which the inhibitors might be acting on different stages in the response mechanism.

A series of experiments was carried out in which the eggs were first placed directly from the ovary either into normal sea water saturated with CO_2 or into a

Table V

Experiments indicating no requirement for magnesium ions

Eggs shed directly into normal sea water	Eggs shed directly into Mg-free sea water	Eggs shed directly into citrate and then, at 6–7 minutes, transferred to: Normal s. w.	or Mg-free s. w.
100	91	100	100
100	98	100	100
100	100	100	97

(Figures indicate % eggs showing nuclear breakdown.)

citrate solution, and then, after 8–10 minutes in one of these solutions, were removed to the other inhibiting solution. Examination showed that nuclear breakdown would be prevented no matter in what sequence the inhibitors were applied. These facts in themselves give virtually no insight into the mechanism of activation, though the information obtained was useful for later interpretations.

A further series of seven experiments proved to be more revealing. If the eggs were shed initially either into 0.53 *M* NaCl (non-inhibitory) or into normal sea water, allowed to remain for three minutes (at 21° C.) and then removed either to normal sea water saturated with CO_2 or to a citrate solution, nuclear breakdown was inhibited only in those eggs in CO_2-saturated sea water.

The results of these experiments suggested a tentative hypothesis which was substantially confirmed and elaborated by later experiments. The data suggest that calcium must perform its (yet to be described) function during the first three minutes after the initial stimulus and that after this function is fulfilled, calcium is no longer needed—since citrate can no longer inhibit. Allen (personal communication) has also shown, in Spisula eggs, that the activation process is sensitive to the lack of calcium during the first four or five minutes after the stimulus is applied. The inhibition by CO_2, both by immediate immersion and immersion after three minutes, presented a more puzzling problem—though not an insoluble one. The immediate inhibition can be explained in at least two ways. CO_2 could either inhibit the release of calcium from binding (possibly in the cortex) or it might prevent calcium from performing any function once it had been released. (It is doubtful that CO_2 inhibits calcium release. In fact, later data indicate just the opposite—that CO_2 produces a release of calcium from binding with protein, for it is expected that in the presence of a sufficient concentration of CO_2 the protein involved is on the acid side of its isoelectric point and is probably no longer capable of binding cations.) Furthermore, CO_2 would appear to inhibit some step in the process that follows both calcium release and the immediate effect that such released calcium produces. This is evident from the fact that calcium apparently completes its function during the first three minutes following the initial stimulus and yet CO_2 is still capable of inhibiting after this time. (These relationships will be more clearly demonstrated by the evidence presented in the next section.) The release of calcium that has been mentioned has not been demonstrated directly for the Chaetopterus egg, though it has been shown for other forms (Mazia, 1937 and Örström and Örström, 1942). The evidence for the existence of this phenomenon in Chaetopterus eggs has been shown indirectly by Wilson and Heilbrunn (1952) and is suggested here by the time relationships of cortical liquefaction (at about two minutes after the eggs have been shed) and the functioning of calcium (at about three minutes after the eggs have been shed).

After the above experiments were performed, a serious criticism came to mind. If there were a significant difference in the rate of penetration of citrate and CO_2 into the cells, many of the conclusions based on the data (regarding time of action) from this entire section would not be valid. For example, the fact that CO_2 could inhibit nuclear breakdown following three minutes immersion in sea water, while citrate could not, might be a result of the ability of CO_2 to penetrate more rapidly and thus affect a reaction that was still sensitive; whereas if the citrate were slower in entering the cell, the reaction might be complete before citrate could be effective.

Fortunately, however, later experiments (see next section) indicate that the general conclusions are substantially correct.

Evidence for the existence of a calcium-activated component

In the previous section, the suggestion was made that after calcium is released from the cortex and during the first three minutes after the eggs leave the ovary, some factor or system—not well defined—was acted upon (or activated) by calcium. This view was based on some indirect evidence. However, as was intimated, some much more conclusive data are available to establish the fact that calcium activates another component.

The experiments to be described were made feasible by an earlier observation that if the eggs were shed into a solution of 0.53 M NaCl saturated with CO_2, not only would nuclear breakdown be inhibited but if the eggs were left in the solution long enough, the calcium of the eggs would leak out. Presumably CO_2 caused calcium to be released from binding with protein, and calcium ions were then able to diffuse out of the cell to a region of lower concentration, there being none in the external medium.

(Examination of Figure 1 will be helpful in understanding the description of the experiments to follow.) Two parallel series of experiments were run. In the first, eggs were shed directly into CO_2-saturated 0.53 M NaCl and allowed to remain in that solution for 12 minutes, during which time nuclear breakdown was inhibited and presumably the calcium of the eggs diffused out into the environment. After 12 minutes some of the eggs were transferred to 0.53 M NaCl (dish A in Fig. 1) and some other eggs were transferred to a solution made up of 7 parts of 0.53 M NaCl and 3 parts of 0.30 M $CaCl_2$ (dish B). If the idea presented above is correct, then nuclear breakdown could not occur in dish A—calcium having been lost before activation was effected. On the other hand, nuclear breakdown would be expected to occur in dish B since calcium was returned to the cell and again made available for activation. In the parallel experiment, eggs were shed directly into 0.53 M NaCl and allowed to remain in that solution for three minutes, during which time calcium would be released and functioning. After three minutes the eggs were placed in CO_2-saturated 0.53 M NaCl, as in the above experiment, and nuclear breakdown was inhibited during the time that the eggs remained in that solution. The eggs were left in this latter solution for 12 minutes, during which time, again, the calcium should have leaked out of the cells. At the end of the 12-minute period some of the eggs were placed in 0.53 M NaCl (dish C) and other eggs were placed in a solution composed of 7 parts of 0.53 M NaCl and 3 parts of 0.30 M $CaCl_2$ (dish D). According to the hypothesis, nuclear breakdown is expected to occur in both latter instances with calcium present or not, since calcium activation should have occurred during the first three minutes in 0.53 M NaCl. Therefore, it is expected that nuclear breakdown would result in both dishes C and D. The predictions for dishes A, B, C, and D were realized. In a typical experiment (in all, four experiments were performed) the following results were obtained:

Dish	% nuclear breakdown
A	22
B	98
C	93
D	88

The critical feature to note is the difference between A and C, though both were devoid of calcium. This evidence is considered as demonstrating that calcium participates in the activation of some component during the first three minutes following the release of the egg from the ovary, and that this calcium-activated factor no longer requires the presence of calcium as it acts more directly on the nucleus. (The process is in this way analogous to the activation of prothrombin to thrombin in blood clotting—a reaction which is also dependent on the presence of calcium. Also, as in the cellular process above, following the activation of thrombin, calcium may be removed from the medium without inhibiting the subsequent reactions in the clotting process.)

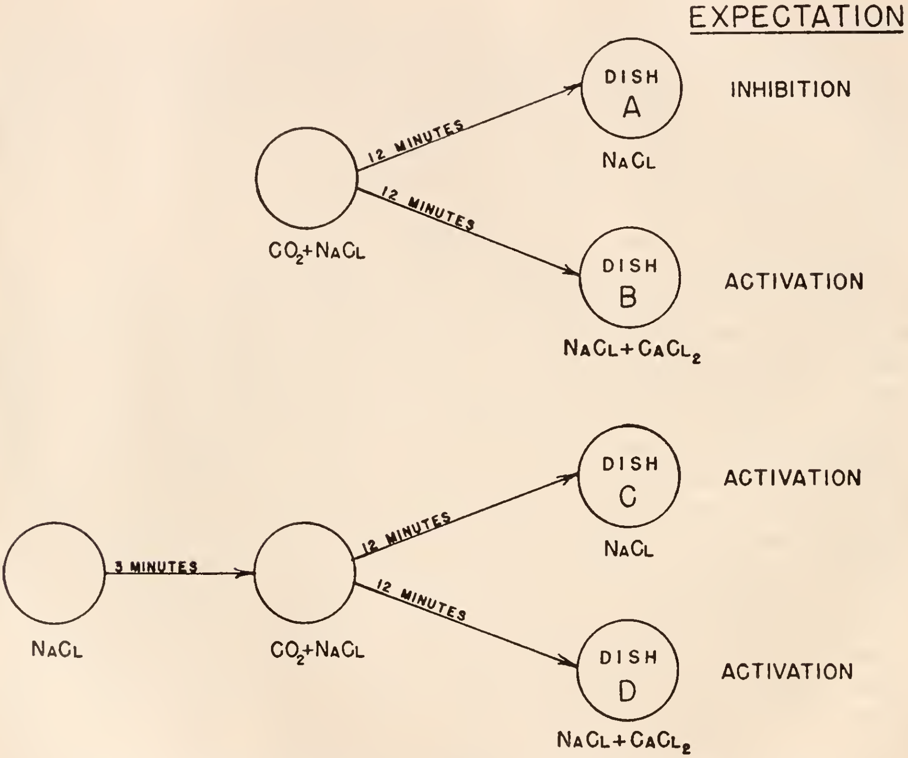
EXPECTATION
DISH A
NaCl
INHIBITION
12 MINUTES
12 MINUTES
CO_2+NaCl
DISH B
$NaCl + CaCl_2$
ACTIVATION
DISH C
NaCl
ACTIVATION
3 MINUTES
NaCl
12 MINUTES
12 MINUTES
CO_2+NaCl
DISH D
$NaCl + CaCl_2$
ACTIVATION

FIGURE 1. Experiment showing the existence of a calcium-activated component. See text for experimental details.

It can be seen that calcium functions at approximately the same time, or shortly after, the cortex loses its rigidity. This evidence suggests that the two phenomena are closely associated and that, presumably, calcium is released from binding in the protoplasmic cortex—resulting in the loss of rigidity. Admittedly, it is impossible to state unequivocally that the *released* calcium is responsible for the activation resulting in the breakdown of the nucleus, since it is possible that the amount of free calcium ions present before stimulation was adequate for nuclear breakdown to have occurred. However, there is probably very little free calcium present in the unactivated egg protoplasm. This is certainly true for the sea urchin egg (Mazia, 1937).

Nature of the calcium-activated component

Since the calcium-activated factor may be acting directly upon the nuclear membrane, which is presumably protein or lipoprotein in character, at least three possibilities as to the nature of the factor were envisioned. (1) Conceivably the activated factor could be acting as a reducing agent to convert S-S linkages to -SH radicals in the protein, thereby leading to a dissolution of the architecture of the membrane. Experiments utilizing -SH and S-S compounds, as well as reagents affecting these groups, established that this probably could not be the mechanism operating. Several reagents were used but most of the experiments were performed with glutathione, cystine and p-chloromercuribenzoic acid, none of which behaved as would be expected from the above hypothesis. (2) It is possible, assuming the presence of lipid in the membrane, that a lipase digests part of the structure and thus breakdown of the nucleus results. This possibility has not been tested. (3) The most appealing hypothesis appears to be that the calcium-activated factor is a proteolytic enzyme.

The foundations for the third view are as follows:

(a) Calcium is known to activate, or participate in the activation of, some proteases. (However, this also applies to some other enzymes, including lipases.)

(b) The nuclear membrane is probably mostly protein and appears to be dissolved during maturation.

(c) Yamane (1930) has reported some experiments which suggest a protease participation in some phase of the maturation mitosis of mammalian ova. Bohus Jensen (1948) and others have also described a certain degree of "nuclear activation" of sea urchin eggs by commercial trypsin.

a. Behavior of trypsin

In order to ascertain if a proteolytic enzyme is capable of producing nuclear breakdown, experiments were carried out utilizing crystalline trypsin (obtained from Worthington Biochemical Laboratory).

(1) Eggs were shed directly into a citrate solution in order to remove the calcium from the cells. After 8 minutes in that solution, some of the eggs were placed in a 0.02% trypsin solution made up in Ca-free sea water, and some eggs were placed in Ca-free sea water without trypsin. The first four items in Table VI show the results of this type of experiment. In every case, though nuclear breakdown took as long as 20–25 mintues for some eggs as compared to the normal time of 7–8 minutes, the eggs which were placed in the trypsin solution gave a substantially higher percentage of nuclear breakdown—though no significant amount of calcium was present in the eggs.

(2) As a check against any possible contamination of the trypsin preparation, another set of experiments was performed in which a trypsin inhibitor was used. In these experiments, following the immersion in citrate for 8 minutes, some of the eggs were transferred to Ca-free sea water, some of the eggs were transferred to 0.03% trypsin in Ca-free sea water, and some were placed in 0.03% trypsin in Ca-free sea water to which was added crystalline soybean trypsin inhibitor (obtained

from Worthington Biochemical Laboratory) to make a 0.03% concentration of that substance. The results of this experiment are shown in the last four items in Table VI. It can be seen, from the figures in the right hand column, that the inhibitor completely blocked the action of trypsin in producing nuclear breakdown. Apparently, then, it was the action of the enzyme itself which was responsible for the breakdown of the nucleus.

(3) Since trypsin is a fairly large molecule, there was some doubt as to whether the enzyme was actually entering the cell. Possibly trypsin was simply acting on the surface to activate, or release, some factor which would act more directly on the nuclear membrane. To determine if trypsin actually penetrates into the cell, trypsin solutions were made up in acid Ca-free sea water (pH 3.5–4.0) and then tested. Following the same procedures as in (1) above, three experiments showed that eggs placed in trypsin in acid media produced substantially as high a percentage of nuclear breakdown as those eggs placed in trypsin in ordinary Ca-free sea water (pH 8.0–8.2). Since, under these circumstances, trypsin is effective at an external pH at which it can not perform proteolysis, the data suggest that the enzyme is entering the cells where the cytoplasmic pH is relatively unaffected by the external hydrogen ion concentration.

TABLE VI

Effect of trypsin on nuclear breakdown

Exper. No.	Concentration of trypsin	% Eggs showing nuclear breakdown in:		
		Ca-free sea water	Trypsin	Trypsin and inhibitor
1	0.02%	31	49	
2	0.02%	5	34	
3	0.02%	5	57	
4	0.02%	4	37	
5	0.03%	10	65	8
6	0.03%	8	81	10
7	0.03%	2	26	3
8	0.03%	9	34	10

Thus we see that trypsin can act within the cell to produce nuclear breakdown in the *absence* of calcium. Consequently, the enzyme appears to be performing the same function as the aforementioned calcium-activated component. Encouragement for these views is offered by the recent observation of Callan (1952) that trypsin is capable of dissolving the membrane of isolated nuclei of Triturus oöcytes.

b. Protease of the eggs

Encouraged by the results of the trypsin experiments, attempts were made to isolate and characterize the intracellular protease—if present. The results to date are preliminary and should only be considered as suggestive

All the eggs from approximately 5 worms were shed into about 35 ml. of Ca free sea water—the process required about four minutes. After mixing to insure equal distribution, the suspension was divided into two parts of 15 ml. each. One portion was frozen immediately in a bath of solid CO_2 and methyl Cellosolve (at ca. $-75°$ C.). The eggs in the other fraction were centrifuged and re-suspended in 15 ml. of normal sea water and allowed to stand for another 8 minutes, after which time (about 14 minutes after the initial shedding) that suspension was also frozen. The two preparations were then dehydrated by freeze-drying. The dried preparations were re-suspended in 5 ml. of distilled water each and allowed to stand for two hours in a refrigerator at 6° C. After two hours in the refrigerator, the suspensions were centrifuged and the supernatants saved.

The two supernatants were then tested for proteolytic activity by the method of Anson (1938). Four minutes after the Folin-Ciocalteau color reagent was added. the optical density, at 700 $m\mu$, was determined with a Coleman spectrophotometer. The results of two experiments are given below; data are expressed in arbitrary units:

	Preparation	Proteolytic activity
Experiment I	Frozen early	11
	Frozen late	− 2
Experiment II	Frozen early	19
	Frozen late	− 2

While the experiments are only preliminary, some interesting possibilities are suggested. It appears that the extracts of the eggs that were frozen about 5 minutes after shedding have a substantial proteolytic activity. This activity would result, presumably, in part from activation before freezing and in part from activation of the dried preparation upon re-suspension in water—the calcium content of the egg material probably being sufficient to produce some protease activation. On the other hand those eggs which had been frozen about 14 minutes after shedding had virtually no activity—the negative figures may be insignificant. The lack of activity in this case can be regarded as the result of a natural protective mechanism of the cell, which would come into play to block further proteolytic digestion before it could act on components of the cell other than the nuclear membrane. This could prevent a possible autolysis. Heilbrunn (1952, p. 654) has considered this type of eventuality and suggests that the phenomenon may be the result of some sort of cellular homeostasis. Thus, in this particular instance it is possible that, following nuclear breakdown, the activated protease could liberate heparin, or a like substance, from binding to protein. (The feasibility of this mechanism has been demonstrated by *in vitro* experiments utilizing trypsin to free heparin from a complex with protamine (Kelly, 1951).) This heparin-like compound would then, presumably, be capable of blocking further proteolysis, and this idea finds support from the evidence showing that heparin is capable of preventing digestion of proteins by trypsin (Glazko and Ferguson, 1940). Further support of this hypothesis is offered by some *in vivo* observations of Kelly (1950). Kelly, by means of metachromatic staining with toluidine blue, has found that there are "granules" of heparin-like material present in Chaetopterus eggs following nuclear breakdown but he was unable to detect their presence before the dissolution of the nucleus (personal communication).

It has been difficult, thus far, to obtain a suitable extract of the eggs within the first minute after shedding. This is regrettable, since the theory holds that the eggs should have little or no proteolytic activity before calcium activation has occurred and it would be desirable to test this. Nevertheless, if we assume that the calcium-activated factor behaves as postulated here, it would not be conclusive proof that this was the system leading to nuclear breakdown in the cell. Direct proof would come from a demonstration that the isolated factor could act on its "natural" substrate—namely, the isolated nuclei. The few experiments that have been performed with this in mind have yielded no information since it has been impossible, as yet, to isolate the nuclei in any condition approaching a reasonably normal physiological state.

Conclusions

From the evidence presented here, the following conclusions have been provisionally drawn regarding the process of activation and nuclear breakdown in the Chaetopterus egg:

1. Within the animal the eggs are "primed" for activation, but some inhibitor is present in the ovarian environment which presumably blocks the release of calcium from the protoplasmic cortex.
2. Following the liberation of the eggs from the animal, calcium ions are released —probably from binding with protein or lipoprotein in the cortex of the cell.
3. Within three minutes after the initial stimulus the free calcium ions activate a system, which is thought to involve a proteolytic enzyme.
4. This activated system then acts directly on the nucleus and produces a dissolution of the nuclear membrane.
5. Immediately following nuclear breakdown some protective mechanism comes into play to prevent excess autolysis of the cell, and probably to allow the cyclic events of mitosis to continue.

The above ideas probably represent the simplest possible mechanism that could be operating.

Two critical problems deserve the earliest consideration. First, it is desirable to achieve a better characterization of the calcium-activated system. Second, a verification of the hypothesis requires that we determine whether or not the isolated system will act on isolated nuclei.

Summary

1. A study has been made of activation and nuclear breakdown in Chaetopterus eggs in an effort to elucidate the various steps in the response mechanism.
2. Within two minutes after the immature egg is stimulated there is a liquefaction of the protoplasmic cortex, which occurs as a prelude to the breakdown of the nucleus. The cortical change appears to be associated with a release of calcium ions from that region.
3. Within three minutes after the initial stimulus calcium ions activate a system which acts more directly on the nucleus and which, having once been activated, no longer requires the presence of calcium ions.

4. It was postulated that the system activated by calcium involved a proteolytic enzyme. To test this possibility experiments were run utilizing crystalline trypsin. It was found that trypsin could produce nuclear breakdown in the absence of calcium and that this activity was completely blocked by soybean trypsin inhibitor. Furthermore, when eggs were placed in trypsin solutions made up in acid media (pH 3.5–4.0), nuclear breakdown still occurred, indicating that the enzyme was penetrating into the interior of the cell.

5. Preliminary experiments suggest that there is a protease in the eggs which behaves as predicted. In addition, it appears that once nuclear breakdown is effected, some mechanism comes into play to block further action of the enzyme.

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PHOTOSYNTHESIS AND PHOTOTAXIS IN ULVA LACTUCA GAMETES[1]

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During reproduction many multicellular algae form fertile areas which differ in color from the vegetative portions and from which are ultimately shed diversely pigmented gametes. These color differences may involve large changes in the ratios of the chlorophylls, carotenoids or phycobilins present in vegetative cells, making such algal material useful for studies concerned with the physiological functions of plastid pigments (Carter, Cross, Heilbron and Jones, 1948; Strain, 1951; Haxo and Blinks, 1950).

The physiology of algal gametes themselves is incompletely understood. For example, few observations have been reported concerning the phototaxis and respiratory activity of these specialized cells and the extent to which they have retained the capacity for photosynthesis. In *Fucus vesiculosus,* Whitaker (1931) reported that the olive-green, non-motile eggs are photosynthetically active, whereas the motile, orange antherozoids are inactive, or at best only very slightly active in this respect, but display a very high rate of oxygen consumption (about 5 times that of the unfertilized egg and about 13 times that of the vegetative tissue). Carter, Cross, Heilbron and Jones (1948 and personal communication, 1952) observed that beta-carotene is the predominating pigment of the orange antherozoids in three species of *Fucus* and in *Ascophyllum nodosum;* chlorophyll was not present in *Fucus vesiculosus* in sufficient amount to be either isolated or identified.

In various members of the Ulvales, the physiological and biochemical changes associated with gametogenesis and sporogenesis can be followed in the intact thallus and such studies can subsequently be extended to the free-swimming gametes. In the sea-lettuce, *Ulva lactuca*, the thallus in both diploid and haploid generations is a broad sheet of rather uniform thickness, composed of two layers of cells embedded in a gelatinous matrix. The gametophyte is heterothallic and during reproduction the vegetative cells give rise progressively to "chlamydomonad-type" gametes along the otherwise undifferentiated thallus margins (Schiller, 1907; Carter, 1926; Smith, 1947). It has been reported that division of the vegetative protoplast takes place at night, resulting in the formation of eight biflagellate gametes which are shed early in the morning (Schiller, 1907; Carter, 1926). After fusion of the isogamous gametes, there is a reversal of the light response, the zygotes being negatively phototactic.

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[1] Contribution from the Marine Biological Laboratory, Woods Hole, Massachusetts, the Department of Biology, Johns Hopkins University, Baltimore, Maryland, and The Charles F. Kettering Foundation for the Study of Chlorophyll and Photosynthesis, Antioch College, Yellow Springs, Ohio.

The color differences associated with gamete formation in *U. lactuca* are apparently similar to, but not always as marked as, those reported by Smith (1947) for *U. lobata* found on the coast of California. In the latter species, the fertile portions of the female thalli are olive-green, and the corresponding portions of the male thalli are tan in color. Strain (1951) has attributed the color changes in the *U. lobata* to variations in the proportions of the chlorophylls and carotenes, the fertile portions of the thalli containing five times as much carotene as the grass-green portions. Smith (1947, 1951) reported that *U. lobata* shows a definite fortnightly periodicity in gamete formation and liberation which is correlated with tidal succession. At Woods Hole, Mass., during the summer months fertile *U. lactuca* can usually be found only at intervals at any one location, suggesting a periodic reproduction; however, the extent to which reproduction in this species is under environmental control is not known.

The present investigation is concerned with studies of photosynthesis, respiration, phototaxis, and pigmentation in free-swimming gametes of *U. lactuca*, as well as some corresponding observations on vegetative thalli and fruiting thallus margins.

MATERIALS AND METHODS

Fronds of *Ulva lactuca* L. var. *rigida* Le Jolis (*cf.* Taylor, 1937) which, by the characteristic opacity and color of the thallus margins, showed evidence of incipient reproduction were collected every few days at low tide in the vicinity of Woods Hole during July and August, 1951. They were maintained in individual pans of sea water in the laboratory until the reproductive cells were shed. Not infrequently a fair percentage of the thalli treated in this manner failed to release either gametes or zoospores. Most fronds which did shed were found to be gametophytic, a distinction which could not be made in the field since the fruiting margins of both female gametophytes and sporophytes are similar in color. The gametes were concentrated by phototaxis, collected with an eye-dropper, and in some cases tested for sex according to the method employed by Smith (1947). The gametes were transferred to centrifuge tubes for measurement of packed cell volume.

Comparison of the rates of photosynthesis and respiration of vegetative thalli and unshed fruiting margins were made on freshly cut discs and strips of thallus. Manometric measurements were made at 25° C. in artificial or natural sea water containing 0.016 *M* carbonate-bicarbonate and having an initial pH of 8.5 (Clendenning and Haxo, unpublished). The light source was a 1000 watt air-cooled projection lamp which provided a maximum light intensity of 5400 f.c. at the manometer vessel. The light was filtered through eighteen inches of water. Polarographic measurements of oxygen production were made in saturating light at about 25° C. by the procedure described by Haxo and Blinks (1950).

Phototactic movement was measured in a cylindrical absorption cell (thickness 1.0 cm., diameter 2.5 cm.) provided externally with a vertical cross hair. By alternate illumination of opposite sides of the cuvette, the gametes which had been introduced as a dilute suspension were first collected as a very thin and uniform layer on the glass wall, and then made to cross the cuvette as a sharply defined wave. The time taken for the wave of gametes to move 5 mm. to the midpoint of the cuvette, as determined visually, was taken as a measure of the motility rate. Rate of movement in different spectral regions of equal intensity was measured with the aid of an Eppley thermopile and Farrand interference filters having peak transmissions at about 440, 560, 620, 680 and 700 mμ. White light intensities were measured with a Weston Sunlight Illumination Meter.

The chloroplast pigments were extracted by one-minute exposure to hot water followed by repeated extractions with methanol. Extraction with absolute methanol was continued until the samples were colorless. The extracted pigments were transferred to ethyl ether by the addition of concentrated sodium chloride solution. Water was removed from the ether by chilling and subsequent drying with anhydrous sodium sulfate. The chlorophyll was estimated spectrophotometrically (Model B Beckman) on an aliquot of the total pigment solution, using Comar's equations (1942). The carotenoids were separated from the chlorophyll by saponifying the total pigment extract with 10–20% KOH. Carotenes and xanthophylls were separated by partition of the petroleum ether solution over 90% methanol and were estimated spectrophotometrically in petroleum ether. Total carotenoids were also estimated from extinction measurements made at 445 mμ on the total pigment extract after correcting for chlorophyll absorption at this wave-length. The total carotenoid values obtained in this way were in close agreement with the sums of the separately determined carotenes and xanthophyll contents. The carotenes from the male gametes were fractionated chromatographically on a column of powdered magnesium oxide and identified on the basis of their characteristic absorption spectra in hexane. For purposes of standardization, alpha- and beta-carotene were isolated from carrots and their absorption curves determined with the spectrophotometer employed in this investigation.

Results

The photosynthetic and respiratory activities of equal areas (1.77 cm.^{2}) of vegetative and fruiting portions of *Ulva* thallus which were observed manometrically are shown in Figure 1. Rectangular Warburg vessels containing 7 ml. of bicarbonate-enriched sea water (0.016 *M*) were employed in this experiment. This volume of liquid provided a large reservoir of bicarbonate, but the associated diffusion lag obscured the details of the transitions. Rates calculated from these data refer to the steady state (Table I). Respiration was much higher in the fruiting thallus margins than in the green vegetative tissue, whereas both net and total photosynthesis was lower. The photosynthetic and respiratory activities are expressed in different terms in Table I, which allow comparisons with the data for free-swimming gametes presented in Table III.

The early time course of photosynthesis in vegetative *Ulva* and other aquatic plants has been previously characterized polarographically by Blinks and Skow (1938), the essential features being an initial oxygen gush, followed by an induction period before the main trend of oxygen evolution resumes. In the present experiments fruiting thallus margins consistently exhibited a longer induction period and lower final rates of O_2 production than vegetative parts of the same thallus after identical pre-treatments. In a typical experiment the tissue was held tightly against the electrode with cellophane as in earlier applications, a condition which would magnify the effects of a high respiration rate on photosynthetic induction, and was maintained in dim light for one hour prior to illumination with saturating light. Oxygen production by the fruiting thallus was sluggish and detectable only in the third minute of illumination. Successive light exposures reduced, but did not abolish, the induction period (Fig. 2a, b, c, d) and no suggestion of an oxygen gush was observed. In the vegetative tissue, however, the oxygen gush and induction period were complete within the first 30 seconds and, upon successive exposures to strong light, oxygen production proceeded at its full rate almost instantaneously, in agreement with the earlier findings of Blinks and Skow (1938, Fig. 5; *cf.* Haxo and Blinks, 1950, Fig. 6).

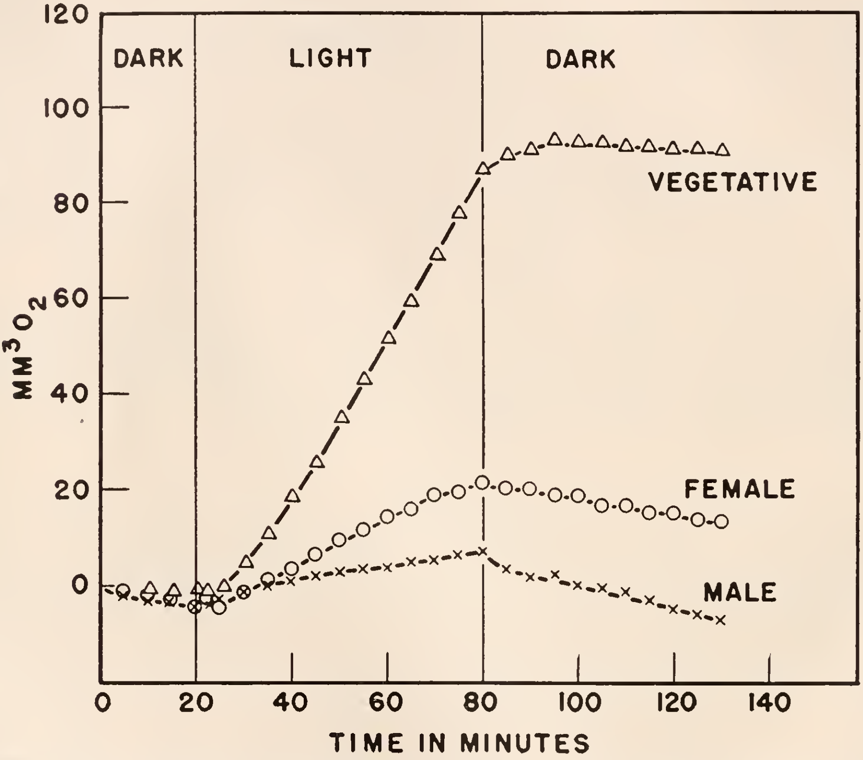

FIGURE 1. Photosynthesis and respiration of vegetative tissue and fruiting thallus margins in carbonate-enriched sea water at 25° C. Area of thallus disc, 1.77 cm^2.

The tissues employed in the foregoing manometric experiment were analyzed for solids, chlorophyll and carotenoids (Table II). The yellowish or male fruiting thallus margin showed the lowest chlorophyll content. The olive-green fruiting margins (in all probability female, but mating responses were not determined in this instance) contained about the same chlorophyll concentration as the vegetative thallus. The carotene contents of both types of fruiting thallus margins were about three times higher than that of the vegetative thallus. The xanthophyll contents of all three samples were essentially the same, so that there was more xanthophyll than carotene in the vegetative tissue, and less xanthophyll than carotene in the reproductive thallus margins. The pigment analyses for the vegetative thalli are

Table I

Photosynthetic and respiratory activity of vegetative thallus and fruiting thallus margins of Ulva lactuca

Gas exchange	Fruiting thallus margin		Vegetative thallus
	Male	Female	
Photosynthesis (corrected)			
mm.^{3} O_2/hr./dm.^{2}	1290	2100	5980
mm.^{3} O_2/hr./gm. fresh wt.	1620	3070	6180
mm.^{3} O_2/hr./gm./solids	7110	12,300	25,400
mm.^{3} O_2/hr./mg. chlorophyll	1320	1720	3490
Respiration			
mm.^{3} O_2/hr./dm.^{2}	755	605	270
mm.^{3} O_2/hr./gm. fresh wt.	950	885	280
mm.^{3} O_2/hr./gm. solids	4180	3530	1180
mm.^{3} O_2/hr./mg. chlorophyll	775	499	160

in reasonable agreement with those reported previously for *U. lactuca* by Seybold and Egle (1938). These authors gave the following values (when expressed as mg./gm. solids): chlorophyll-a, 3.3; chlorophyll-b, 1.5; total chlorophyll, 4.8; carotene, 0.16; xanthophyll, 0.77; total carotenoid, 0.93.

Expressed on a solids basis, the free-swimming female gametes contain twice as much chlorophyll and half as much carotene as the male gametes; the xanthophyll content was the same in both cases (Table III). Comparisons cannot readily be made on the same basis between the pigment content of the free-swimming gametes and that of the vegetative thalli (Table II). It is interesting to note, however, that the ratio of chlorophyll:carotenoid has changed from 8.3 in the vegetative tissue to 2.7 in the female gametes and 1.1 in the male gametes. Strain (1951) has reported that in *U. lobata* a large portion of the increased carotene content of the sexually differentiated margins is due to the gamma-carotene isomer, only a little of which occurs in vegetative thalli. A similar change appears to occur in *U. lactuca.*

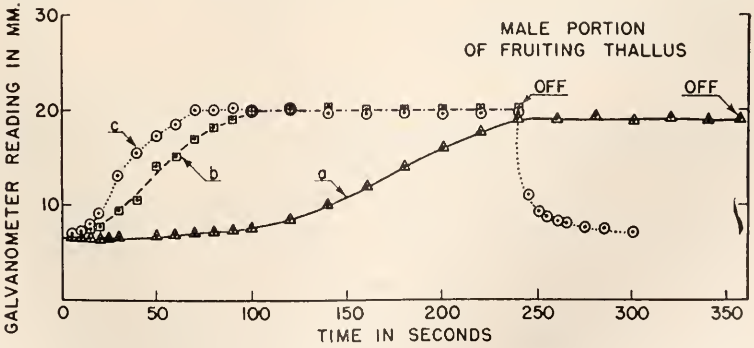

Figure 2. Initial time course of oxygen evolution which was observed polarographically on a strip of male fruiting thallus margins at *ca.* 25° C., after a pre-treatment of one hour in dim light.

TABLE II

Chlorophyll, carotenoid and solids data for vegetative and reproductive thallus margins of Ulva lactuca

Constituents	Fruiting thallus margins		Vegetative thallus
	Yellowish	Greenish	
Fresh wt., mg./dm.^{2}	797	683	967
Solids, mg./dm.^{2}	181	171	230
Chlorophyll, mg./gm. solids	5.41	7.15	7.29
Carotene, mg./gm. solids	1.35	1.49	0.34
Xanthophyll, mg./gm. solids	0.68	0.64	0.54
Sum of carotenoids, mg./gm. solids	2.03	2.13	0.88
Ratio, chlorophyll/carotenoids	2.7	3.4	8.3

Chromatographic and spectral analyses of the abundant carotene fraction present in the male gametes indicated the following composition: 43% gamma-carotene, 32% beta-carotene, 3% alpha-carotene, and 22% unidentified carotene.

The green (♀) and yellow (♂) gametes, whose photosynthesis and respiration are reported in Figure 3 and in Table IV, were actively phototactic at the time, and also showed the normal clumping reaction of opposite sexes on admixture. Respiration of the male gametes exceeded that of the female,[2] and respiration of both types exceeded that of the unshed fruiting margin as well as of the vegetative thallus. Expressed on a solids basis, the respiration of the male gametes was almost thirty times greater than that of the vegetative thallus, and was about fourteen times greater in terms of chlorophyll content.

TABLE III

Chlorophyll, carotenoid and solids data for Ulva lactuca gametes

Constituents	Male gametes	Female gametes
Dry wt., mg./ml. packed cells	96	105
Chlorophyll, mg./gm. solids	14.42	24.0
Carotene, mg./gm. solids	8.20	4.68
Xanthophyll, mg./gm. solids	4.70	4.20
Sum of carotenoids, mg./gm. solids	12.90	8.88
Ratio, chlorophyll/carotenoids	1.12	2.7

Despite their very high respiration rates, both the male and female gametes exhibited net photosynthesis in intense light, the levels of net assimilation being similar in the two kinds of gametes. The corrected or total photosynthesis for the male gametes exceeded that for the female gametes, regardless of the basis used in expressing photosynthetic activity. The corrected rates of photosynthesis which were observed in the gametes were considerably lower than the corresponding rate for vegetative thallus on a chlorophyll basis. The corrected rates of photosynthesis observed in the free-swimming gametes were higher, however, than the rate for vegetative tissue when expressed in terms of solids content.

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[2] The present data are insufficient to establish whether the isogamous male and female gametes of *U. lactuca* differ significantly in this respect.

Photosynthesis by the gametes has been compared manometrically in red and white light (Fig. 4). The rate of oxygen evolution obtained with saturating white light was identical to that observed on exposure of the gametes to the component red light. In contrast to their phototaxis, photosynthesis by the gametes was effectively sensitized by light absorbed specifically by chlorophylls. Their photosynthetic mechanism could be completely saturated by red light which was entirely ineffective in orienting the swimming motion. This experiment does not establish the relative effectiveness of chlorophylls and carotenoids as sensitizers of photosynthesis in the gametes of green algae.[3] However, all earlier studies of vegetative green algae have indicated that light absorbed by the carotenoids is utilized less effectively than light absorbed specifically by chlorophyll (Emerson and Lewis, 1943; Haxo and Blinks, 1950).

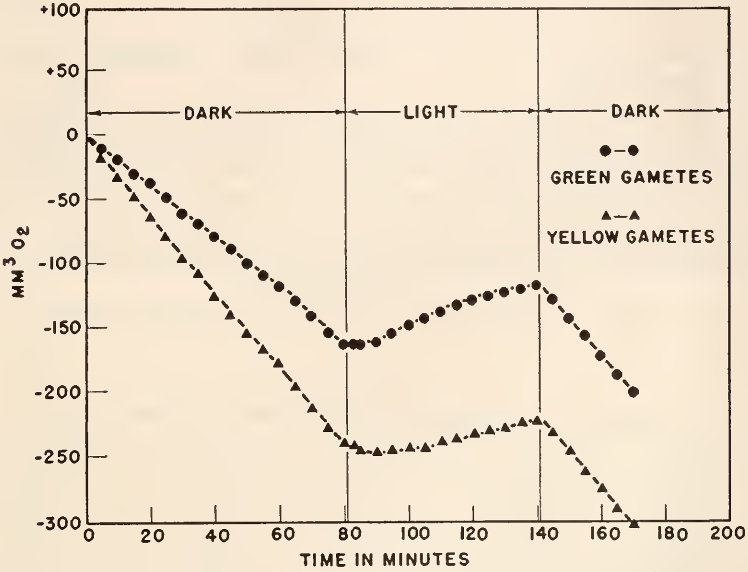

FIGURE 3. Photosynthesis and respiration in male and female *Ulva* gametes in carbonate-enriched sea water at 25° C.; 0.0815 mg. chlorophyll in male, 0.161 mg. chlorophyll in female gametes.

Ulva gametes swim actively in total darkness as well as when illuminated. Thus when gametes are drawn to the side of a cuvette by phototaxis, and are then maintained in darkness, the gametes soon diffuse throughout the vessel by random motion. The same unoriented motility is shown when the gametes are illuminated by light of wave-lengths above 550 mμ. Positive phototaxis of *Ulva* gametes is a response to blue-violet light (ca. 440 mμ). Although blue-violet light orients the

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[3] Unsuccessful attempts were made to determine the photosynthetic action spectra of male margins, employing the available polarographic apparatus which did not permit estimation of the large respiration correction. Upon illumination of this tissue at low and moderate light intensities, either no change in oxygen concentration at the electrode or an actual uptake of oxygen was noted.

TABLE IV

Photosynthetic and respiratory activity of Ulva lactuca gametes

Gas exchange	Male gametes	Female gametes
Photosynthesis (corrected)		
mm. O_2/hr./ml. packed cells	3960	3550
mm. O_2/hr./gm. solids	41,200	33,800
mm. O_2/hr./mg. chlorophyll	2570	1410
Respiration		
$mm.^3$ O_2/hr./ml. packed cells	3380	2750
$mm.^3$ O_2/hr./gm. solids	35,200	26,200
$mm.^3$ O_2/hr./mg. chlorophyll	2200	1090

swimming motion of the gametes, their flagellar activity is apparently not otherwise controlled by light energy.

The positive phototactic response of dilute cell suspensions, as observed by rate of linear movement, increases to a pronounced extent in the range 0 to 50 f.c., but increasing the white light intensity above 100 f.c. has only a small further effect (Fig. 5). The saturating light intensity for photosynthetic activity in *Ulva* gametes (50 $mm.^3$ cells in 5 ml. sea water at 25° C.) is above 2000 f.c. Photosynthesis and phototaxis in *Ulva* gametes thus require quite different light intensities for

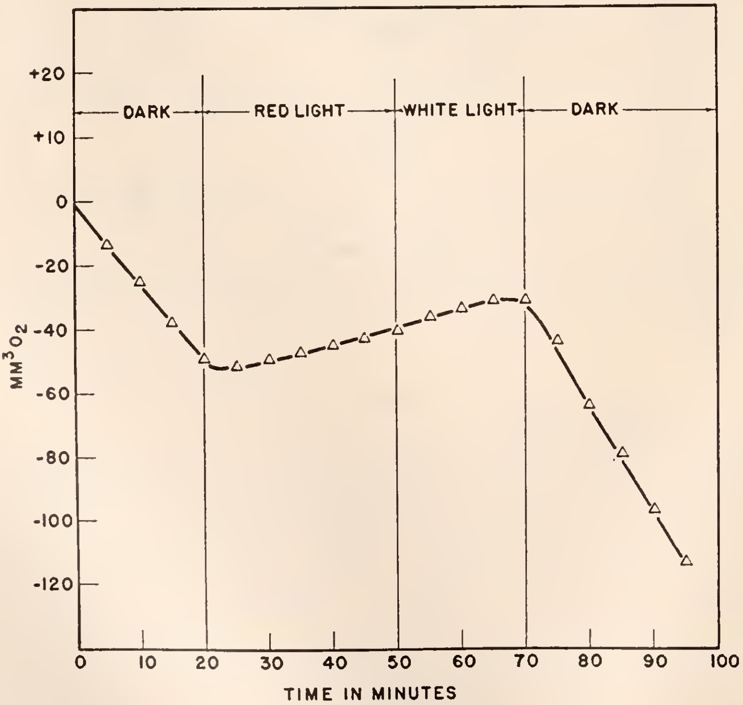

FIGURE 4. Photosynthesis and respiration in female *Ulva* gametes in red and white light, approximately 50 mm^3. gametes in 7 ml. carbonate-enriched sea water at 25° C.

saturation, and also have very different wave-length specificities. Red light is highly effective in sensitizing their photosynthesis although it plays no part in inducing the phototactic response.

The fastest rate at which *Ulva* gametes have been observed to swim towards a strong light source is *ca.* 18 mm. per minute (Fig. 4). The size of the gametes, exclusive of flagella, is *ca.* 7.6 by 3.6 μ (length 6.9–8.3 μ, width 3.5–3.8 μ). From the observed rates of phototactic movement and size of the cells, it is apparent that *Ulva* gametes swim more than two thousand times their body length in one minute.

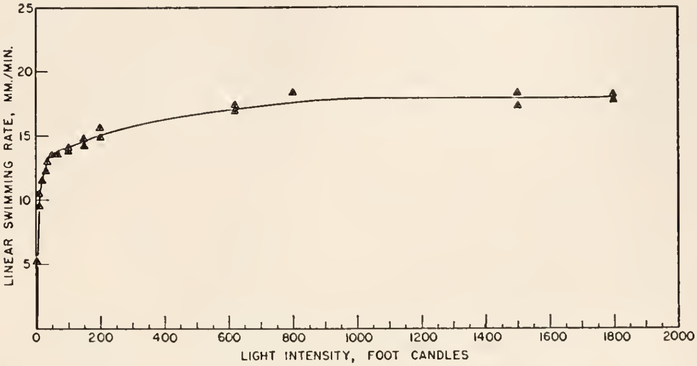

FIGURE 5. Maximum linear movement of *Ulva* gametes at *ca.* 25° C. as a function of light intensity.

DISCUSSION

The manometric measurements of photosynthesis and respiration indicate that gamete formation in *U. lactuca* is accompanied by physiological changes which are detectable prior to the onset of motility and the actual release of the gametes. Most notable is the increase in the rate of oxygen consumption, which begins in the differentiating thallus margins and is further accentuated in the free-swimming gametes. It would be of interest to ascertain whether this increase in metabolic activity is gradual or abrupt, and whether it is associated with specific stages in gametogenesis. Although the entire vegetative thallus of the *Ulva* gametophyte may ultimately give rise to gametes, at any one time gametogenesis is normally restricted to the peripheral margins of the thallus. Fruiting specimens have been collected, however, in which the thallus has been torn internally, leaving the periphery intact. The vegetative area bordering the cut surfaces occasionally has been observed to have undergone color differentiation and ultimately to release gametes. Experimental studies might reveal more fully the conditions under which gamete formation is initiated and maturation of the gametes proceeds.

The pronounced induction period which was observed polarographically in the fruiting thallus margin is attributed to oxygen deficit resulting from the high respiration rate of this tissue, as well as its relative inaccessibility to oxygen while pressed tightly against the platinum electrode. Blinks and Skow (1938) had earlier found for vegetative *Ulva* and other aquatic plants that the induction loss is magnified by lengthy dark anaerobic periods, and may be eliminated by adequate aeration in the dark or by successive exposures to bright light.

The respiration of free-swimming *Ulva* gametes is very much higher than has been observed previously in photosynthetically active cells, and is about 50% higher than that reported by Whitaker for the non-photosynthetic *Fucus* antherozoids. The compensation point of *Ulva* gametes has not yet been determined accurately over a range of temperature and cell densities, but with 50 mm.^{3} cells in 5 ml. sea water at 25° C., the compensating white light intensity was above 1500 f.c. Because of their high respiration rate, the compensation point for *Ulva* gametes is much higher than any of the values which were recently assembled by Rabinowitch (1951).

The orienting effect of blue light on *Ulva* gametes makes it possible to measure their swimming rates quite accurately. The rate of movement which was observed in this way (18 mm. or over 2000 times the cell length per minute) on *Ulva* gametes is probably higher than that of most other motile cells, although few data of this sort are available in the literature. Using the same method we have observed motility rates of up to 16 mm./min. for both positive and negative phototaxis in the green flagellate, *Platymonas subcordiformis;* however, the motility rates observed for several flagellates were much lower. According to Patten (1946) spermatozoa travel 1.5–3.0 mm. per minute whereas Rothschild (1951) has reported the translatory speed of spermatozoa to be *ca.* 12 mm. per minute.

Photosynthesis and phototaxis exhibit such different wave-length specificities and saturate at such different light intensities that they should be regarded as unrelated processes in *Ulva* gametes. This probably represents the situation for flagellated algae in general, since phototaxis in, for example, *Euglena* and *Chlamydomonas* (Mast, 1917), *Volvox* (Laurens and Hooker, 1920), and in the green and red phenotypes of *Dunaliella salina* (Blum and Fox, 1933) has been shown to be limited to the blue end of the spectrum. *Rhodospirillum rubrum* presents a special case since Thomas and Nijenhuis (1950) have reported that the action spectra, saturating light intensities and responses to cyanide and ethyl urethane were the same for photosynthesis and phototaxis in this organism.

Both male and female fertile portions of *U. lactuca* thalli contained more carotene than did vegetative portions of the thallus, being apparently similar in this respect to *U. lobata* (Strain, 1951). In the free-swimming gametes the carotene content of the male gametes was about twice that of the female reproductive cells. High carotenoid concentrations are generally characteristic of male gametes, whereas there is a greater retention of chlorophyll in the female gametes (Karrer *et al.*, 1943; Carter *et al.*, 1948; Strain, 1951). The widespread accumulation of carotenoids in reproductive cells and tissues suggests that carotenoids may play a role in sexual reproduction (Emerson and Fox, 1940; Cook, 1945; Goodwin, 1950), in addition to their probable role as sensitizers of phototaxis (Wald, 1943). Whatever the role of carotenoids in sexuality may be, it seems evident that the abundant accumulation sometimes observed in gametes is in considerable excess of the requirements for this function and may not be related to it. *Allomyces* species accumulate abundant stores of gamma-carotene in the male gametes (Emerson and Fox, 1940); however, Turian (1952) has inhibited the visible production of pigment without apparently altering the capacity for mating. The apparently normal sexual reproduction of albino strains of *Neurospora* (Hungate, 1945) is another case in point.

Photosynthetically active cells always contain at least traces of chlorophyll but the minimal chlorophyll requirements for photosynthesis have not been established with certainty (*cf.* Rabinowitch, 1945, 1951). In investigations of this question, advantages are presented by algal gametes, in that the cells are in a healthy state of high metabolic activity irrespective of their chlorophyll content. Furthermore, by use of several species it might be possible to obtain for study a series containing successively smaller amounts of chlorophyll. In *U. lactuca* the chlorophyll content of the female gametes was almost twice that of the male gametes and yet showed a lower photosynthetic activity in terms of chlorophyll content. A visible color differentiation of about the same degree is found in the gametes of *Bryopsis corticulans* but Blinks (personal communication, 1952) has observed that only the green (female) gametes show photosynthetic activity in excess of the compensation point. As was indicated previously, *Fucus* eggs contain chlorophyll and effect net photosynthesis in strong light, whereas, apparently, *Fucus* antherozoids contain no chlorophyll (Carter *et al.,* 1948) and are incapable of photosynthesis (Whitaker, 1931).

Thanks are due various colleagues in the Botany Division of the Marine Biological Laboratory for help in the collection and handling of the algae used in this study; and in particular Dr. Maxwell Doty for his suggestions and Shirley Trefz for assistance in some of the preliminary experiments.

SUMMARY

1. Fruiting margins of *Ulva lactuca* thallus are characterized by a higher content of carotenoids, as well as by higher respiratory and lower photosynthetic activity, than the corresponding vegetative thallus. Photosynthesis in the male margin was also characterized by a longer induction period than was observed in the vegetative thallus by the polarographic method.

2. The changes in pigment composition observed in *Ulva lactuca* during reproduction are similar to those reported for *Ulva lobata.* The male gametes of *Ulva lactuca* contained twice as much carotene and half as much chlorophyll as the female gametes. The male carotene fraction contained 43% gamma-carotene, 32% beta-carotene, 3% alpha-carotene and 22% unidentified carotene. The xanthophyll contents of the male and female gametes were similar. The chlorophyll/carotenoid ratio was 8.3 in the vegetative thallus, 2.7 in the female gametes and 1.1 in the male gametes.

3. The respiration of the male gametes exceeded that of the female gametes, was almost thirty times greater than that of the vegetative thallus on a solids basis, and was about twelve times greater in terms of chlorophyll content.

4. Both male and female gametes exhibited net photosynthesis in intense white light, but a very high light intensity was required to compensate their respiration, which is the highest that has been reported for cells possessing photosynthetic activity.

5. Positive phototaxis of *Ulva* gametes is a response to blue-violet light, which orients their swimming but does not otherwise control their flagellar activity. Their maximum rate of linear movement is attained at moderate light intensity (*ca.* 100 f.c.). The gametes swim 2500 times their own body length each minute.

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A STUDY OF CHEMORECEPTION IN AQUEOUS AND GAS PHASES[1]

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Although it is generally recognized that chemoreception plays an important part in the orientation, feeding and reproductive activities of many aquatic animals (Jahn and Wulff, 1950; Walker and Hasler, 1949), these animals have been a perplexing group in developing concepts of the physiology of the chemical senses. At first it was even questioned whether presumed chemical sense organs of aquatic organisms could have any function at all, since they were surrounded by a fluid medium rather than a gaseous one (Nagel, 1894). Nagel's negative conclusion, based upon anthropomorphic considerations, has long been discredited by experiments indicating that many reactions of aquatic animals are mediated by receptors which he held to be functionless—*e.g.,* the receptors in the olfactory pits and connected to the olfactory nerves (Parker and Sheldon, 1913; von Frisch, 1924).

Matthes (1924), interested in adaptations of the sensory apparatus to different environments, then raised the question whether the same receptors could mediate reactions to chemical stimuli underwater and on land in the case of the animals which spent a portion of their life cycles in each environment. It was found that *Triton* no longer reacted to chemicals in either environment when the olfactory nerve was cut. The olfactory innervation was traced to the Jacobson's organ (Matthes, 1927) and it was shown that the presumed receptors in the larval and adult animals were histologically very similar to those of other vertebrates. When it was discovered that a sheet of mucus, variable in quantity and distribution according to the physiological state of the animal, overlay the olfactory receptors (Murphy, 1931; Leasure, 1939) the logical assumption that chemical stimuli went into solution in the mucus layer abridged the usefulness of the previous observations on vertebrates for understanding what specializations, if any, of the receptors existed for functioning in gas and liquid phases. Schaller (1926) avoided this difficulty by studying amphibious beetles which have chemoreceptors on mouthparts accessible for ablation experiments, and which could be tested both underwater and with a dry cuticular surface in air. Conditioned reactions of *Dytiscus* to coumarin and synthetic musk, either underwater or in air, ceased after removal of the flagellar portion of the antennae and the maxillary palpi. Acid, salt, and sugar were similarly detected by receptors on the labial palpi and the inside of the mouth. It was concluded that different groups of receptors were specialized for perception of either of two types of chemical stimuli—those which are "odor-substances" for man (irrespective of physical state of the stimulus), or those compounds which are

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"taste-substances" for man, with secondary specializations for perceiving particular modalities of taste substances, such as acid, salt, sugar, and bitter compounds, since these compounds are discriminated by *Dytiscus*. However, conflicting conclusions based upon experiments with the same and related species of insects were presented by Ritter (1936) and Bauer (1938). Although accepting Schaller's main concept about the types of physiological specializations to be found in chemoreceptors, they did not agree on the location or morphology of the receptor groups sensitive to "odor-substances," "taste-substances," or the various modalities of the latter group. Ritter (1936) reported that the antennae of *Hydrous* completely lacked chemoreceptors and that heliotropin and skatol stimulated receptors on the tips of the maxillary palpi; the receptors of the labial palpi were reported to be sensitive only to acid. Bauer (1938) failed to prevent reaction to non-acidic stimuli by removing the maxillary palpi. Nor was agreement reached on the morphological appearance of the variously located and specialized receptors.

In a recent review, Dethier and Chadwick (1948) discussed possible sources of conflicting data in the experiments cited above (such as the lack of quantitative control of the stimulus) and have concluded that the relationship between the physical state of a chemical and its effect on chemoreceptors is unknown. They further conclude that this relationship should be explored not only to understand what specializations, if any, differentiate receptors functioning in gas and liquid phases and the sensitivity to various modalities of stimuli in each phase, but also to determine whether the same limiting mechanism operates in both phases. The aim of the present work is to determine, first of all, whether the same set of receptors mediates reactions to chemical stimuli administered in gas and liquid phases and whether different groups of receptors are specialized for perception of particular modalities of stimuli in either phase. The question of the fundamental limiting mechanism in each phase is later discussed.

Materials and Methods

The experimental animal was an amphibious beetle, *Laccophilus maculosus* Germar, chosen because of its dual air-water habitat, accessible mouthparts, and availability. It has been shown (Hodgson, 1951) that *Laccophilus* possesses much lower reaction thresholds to a wide variety of chemical stimuli than those reported for other insects. Since the relations between molecular structure, chemical properties, and relative stimulating effectiveness of compounds included in several series of organic and inorganic chemicals determined for *Laccophilus* are essentially identical with the findings obtained in experiments with *Phormia* (Dethier and Chadwick, 1948), *Balanus* (Cole and Allison, 1930) and other terrestrial and aquatic invertebrates, it was concluded that at least the limiting mechanisms of chemoreception in *Laccophilus* were not aberrant and might yield information of general significance. The beetles were collected at the Fish Hatcheries Experiment Station of the United States Fish and Wildlife Service at Leetown, West Virginia. They were obtained in large numbers both in shallow water of the fish rearing ponds and in dry grass near the ponds. The methods used for maintaining stocks of these animals have been previously described (Hodgson, 1951). Every precaution was taken to standardize feeding and handling of the beetles and after being once used in the experiments the beetles were discarded.

The technique for determining threshold concentrations of pure chemicals administered in the gas phase or in the liquid phase is essentially the same, although a different apparatus is required for each of the two types of test. The over-all procedure is to apply moving, unmixed streams of air or water, containing known concentrations of the chemical to be tested, to groups of beetles and determine the percentage of beetles which react to the chemical by moving into the control area of the apparatus which is exposed to a moving stream of air or water identical in all respects except that the test compound is absent. Technical details of the construction and operation of the apparatus for determining threshold concentrations of chemicals in aqueous solution have been fully presented (Hodgson, 1951) and hence will not be repeated, since the method used here was identical. The apparatus for determining threshold concentrations of gases was a slight modification of the olfactometer described in detail by Dethier and Yost (1952), and used by these investigators in experiments with the blowfly, *Phormia.* In brief summary of this method, animals to be tested are placed in a cage through which pass two streams of air, one containing a known concentration of the gaseous chemical being tested. Before the experiment, the animals are distributed randomly in the two halves of the cage exposed to the experimental and control streams of air. Those reacting to the gas being tested move to the control side of the cage. The percentage doing so is determined by photographs taken during the run. With *Laccophilus,* it was found that the best results were obtained using a smaller cage (4″ × 2″ × 3/8″) than was used with *Phormia.* This narrow cage was placed horizontally in the apparatus so that the air stream passed through it from top to bottom, since *Laccophilus* did not climb the vertical walls of the cage as readily as did *Phormia.* Fifty unanesthetized beetles, completely dry on the external surface of the cuticle, were placed in the cage at one time. Since the number of beetles reacting is equal to the difference between the average number of beetles remaining on the experimental side of the cage and the average number originally on that side (about 25 beetles), the formula used to obtain the percentage reaction to any particular concentration was as follows: $\% \text{ reaction} = \frac{25 - x}{25}$, where x is the number of beetles remaining on the exposed side of the cage. Aside from the slight modification of the test cage construction, this procedure was identical with that described by Dethier and Yost (1952), and their report may be consulted for technical details of the apparatus, sample calibration curves, etc.

With either aqueous or gas phase tests, doubling concentrations of the chemical stimulus are administered. The percentage of beetles reacting at each concentration is converted into probit units by the method of Bliss (1938). This conversion merely transforms the typical sigmoid dosage-reaction curve to a straight line, in which the 50% reaction point corresponds to a probit of 5. The direct relationship between the logarithm of the molar concentration of the stimulant and the percentage of beetles reacting is shown in Figure 1. The data used in this plot were selected simply because the thresholds in these two particular cases fell within a range convenient to include on a single graph. From such a plot, the 50% reaction point (threshold) can be read directly. The standard error of the threshold is obtained by the method of Miller and Tainter (1944). The tabulated results presented below are derived from data on 69 individual beetles tested in three different runs in the liquid phase, and from data on 150 individuals tested in three different runs in the case of the gas thresholds. This difference in numbers results from the different sized populations convenient to test in the different sized reaction chambers of the two types of apparatus. An analysis of the variance between reactions of the three different groups of beetles tested at each concentration of stimulus showed no significant difference between the reactions of the three groups in either phase and the data were therefore combined and treated as though derived from a single homogeneous population.

When beetles were to be operated upon, they were anesthetized with carbon dioxide and lifted onto clay blocks, having depressions into which the animals fit with their ventral sides uppermost. A broad rubber band around the block covered the posterior halves of the beetles and held them in position during the operations.

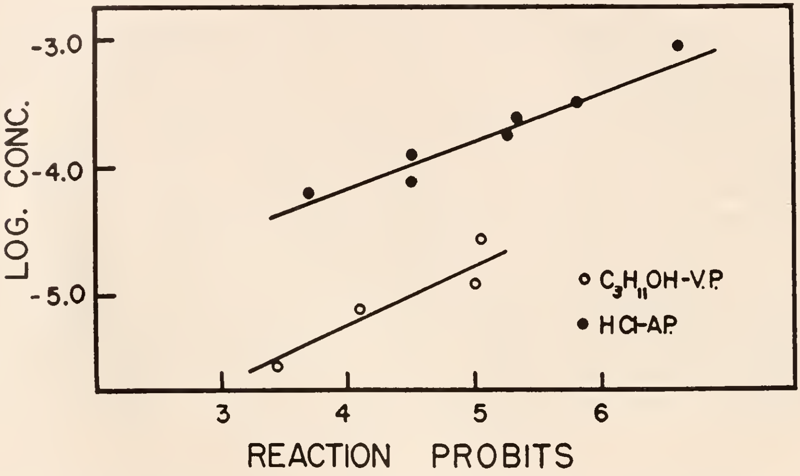

FIGURE 1. Relationship between percentage reaction and quantity of stimulating chemicals. Data in this plot obtained from experiments on unoperated beetles exposed to 1-pentanol in the vapor phase and HCl in the aqueous phase.

Watchmaker's forceps and iridectomy scissors were used to grasp and remove whatever portions of the head appendages were desired. The point at which the appendages were severed when they were to be completely removed is indicated in Figure 2. Other operations were performed as noted blow. The effects of the anesthetic wore off within five minutes and the operated beetles seemed to have no difficulty swimming or flying.

To restrict the investigation to a manageable size, the chemicals used were restricted to 1-pentanol, HCl, and NaCl. The first two compounds can be conveniently worked with in the vapor phase and enough data on their thermodynamic and other properties exist to permit comparisons of possible mechanisms of action in the two phases. The latter two compounds represent classic modalities (acid and salt) of taste stimuli which were the source of the conflicting data cited in the introduction. It should be noted that although it would have been desirable to include a sugar among the aqueous phase stimulating compounds, *Laccophilus* does

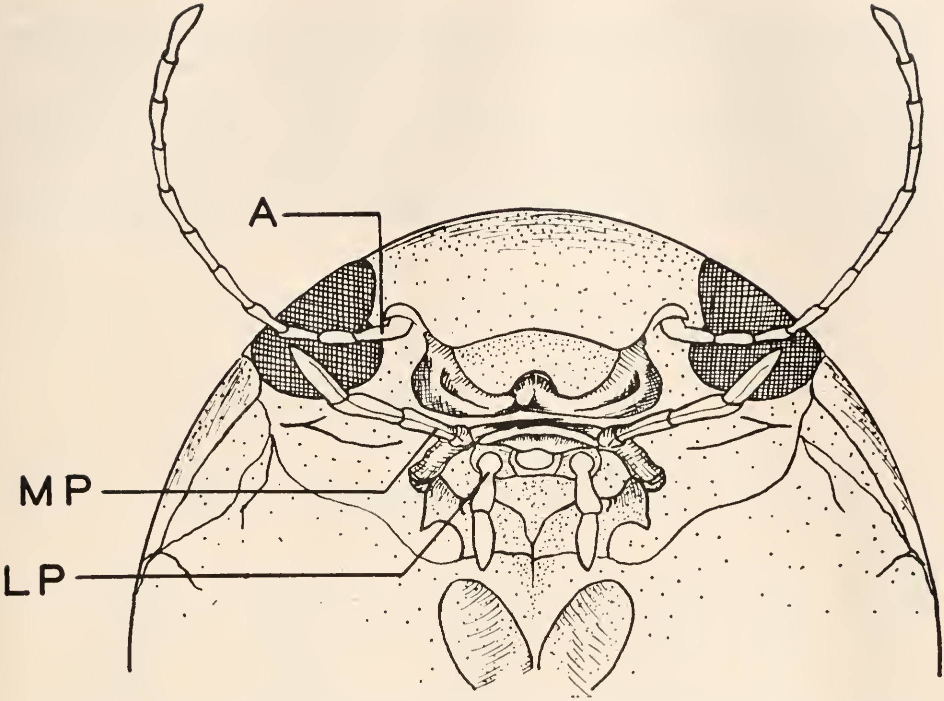

FIGURE 2. Ventral view of *Laccophilus* head, showing antennae and palpi. The pointers indicate the positions near the base of each appendage where they were severed if they were to be entirely removed.

not react to aqueous solutions of sucrose when tested by this method with the highest concentration it is possible to use in the apparatus and still maintain normal rates of flow (approximately one molar sucrose).

RESULTS

1. *The identity of receptors mediating reactions in the gas and liquid phases.* (Threshold data are summarized and compared in Tables I and II.)

Beetles were exposed to 1-pentanol and HCl in both phases and thresholds determined for the normal (unoperated) animals. As noted above, data from three groups are treated as one homogeneous unit to derive each threshold value. The results are given in Table I, Columns 1A and 1B. Then the antennae were completely removed from another series of animals and a similar run made in both phases (Table I, Columns 8A and 8B). Since it appeared that a complete loss of sensitivity to the gaseous stimuli, within the range of concentrations it was possible to test with the present apparatus, was suffered by beetles with antennae removed, it was not necessary to go on to other mouthparts in attempting to localize the receptors mediating reactions to gases, and a more precise localization of the gas phase receptors on the antennae was sought.

After several preliminary experiments, it was found that the smallest area which it was possible to cut accurately from the distal ends of the antennae was one-half of the distal segment. When the extreme distal half segments of the antennae were removed from 150 beetles, the animals gave no reaction to either 0.0003 molar pentanol, or to 0.0002 molar HCl, administered as gases. These were the highest concentrations of these compounds which it was possible to administer with the olfactometer. Therefore, it was concluded that the chemoreceptors mediating reactions to gaseous stimuli are located on the distal halves of the distal segments of the antennae.

TABLE I

Aqueous and vapor phase thresholds of Laccophilus

	Threshold (molar concentration evoking reaction from 50% of beetles) ± standard error. Appendages listed at top of columns indicate parts removed.								
	1. Control (unoperated)	2. MP LP	3. MP LP ½S − A	4. A LP	5. A LP ½S − MP	6. A MP	7. A MP ½S − LP	8. A	9. A MP LP
A. Aqueous phase 1-pentanol	0.0019 ± 0.0003	0.0015 ± 0.0007	*	0.0082 ± 0.0022	*	0.0076 ± 0.0010	*	0.0050 ± 0.0007	*
HCl	0.0014 ± 0.0006	0.0019 ± 0.0010	*	0.012 ± 0.005	*	0.0090 ± 0.0008	*	0.0073 ± 0.0006	*
NaCl	0.17 ± 0.03	0.18 ± 0.08	*	0.78 ± 0.13	*	0.73 ± 0.19	*	0.48 ± 0.09	*
B. Vapor phase 1-pentanol	0.00015 ± 0.00008	0.00027 ± 0.00011	—	—	—	—	—	*	—
HCl	0.00012 ± 0.00003	0.00014 ± 0.00005	—	—	—	—	—	*	—

Abbreviations and symbols used: A, antennae; MP, maxillary palpi; LP, labial palpi; S, distal segments; *, no reaction to highest concentration tested.

To see if the same area was involved in mediating reactions to these chemicals in aqueous solution, the distal halves of the distal segments of the antennae were removed from 69 other beetles, previously deprived of palpi—which, it will be shown, bear other chemoreceptors—and these beetles exposed to pentanol and HCl (Table I, Column 3A). These beetles no longer reacted, even to stimuli as concentrated as 0.1 molar pentanol and 0.5 molar HCl, which are, respectively, fifty and three hundred times as concentrated as would be necessary to stimulate 50% of the unoperated animals.

At first it was not known where the other receptors mediating reactions to chemicals in the liquid phase were located. On the basis of Schaller's experiments it was expected that they would be located on the palpi and in the mouth cavity.

Accordingly, the antennae and labial palpi were removed from a group of beetles, leaving only the maxillary palpi. The thresholds of this group were significantly higher than for the normal animals (Table I, Column 4A; Table II, Column 3). Removal of the distal half of the distal segment of the maxillary palpi in another group of beetles, previously deprived of antennae and labial palpi, abolished reactions to chemical stimuli at the highest concentrations mentioned above (Table I, Column 5A). Hence, it was concluded that only the distal half-segments of the maxillary palpi bear chemoreceptors. Further operative subdivision of this segment was not technically possible.

Next, the antennae and maxillary palpi were completely removed from a group of beetles, leaving the labial palpi. The threshold again increased significantly as compared with the normal animals (Table I, Column 6A; Table II, Column 4), but did not differ significantly from the threshold of the receptors on the maxillary palpi alone (Table II, Column 7), or from the threshold of the combined maxillary and labial palpi (Table II, Column 6). Removal of the distal segments of the labial palpi from a group of beetles previously deprived of their antennae and maxillary palpi abolished all reaction to the highest concentrations of chemical stimuli tested (Table I, Column 7A). In the case of the labial palpi, too, the chemoreceptors must be on the distal segment, although the small size of this segment precluded any further localization by ablation techniques.

TABLE II

Comparison of thresholds of receptor groups in Laccophilus

Compound	Receptor areas or operative conditions being compared. The upper figure of each set is the difference in molar threshold, and the lower figure the standard error of the difference							
	1. Normal; minus A	2. Normal; A	3. Normal; MP	4. Normal; LP	5. MP; MP+LP	6. LP; MP+LP	7. LP; MP	8. Normal; A (gas phase)
1-pentanol	0.0031 0.00095 *	0.0005 0.0011	0.0063 0.0022 *	0.0057 0.00105 *	0.0032 0.0023	0.0026 0.0013	0.0006 0.0024	0.00012 0.0080
HCl	0.0059 0.00085 *	0.0005 0.0012	0.0096 0.005 *	0.0076 0.0010 *	0.0057 0.0050	0.0017 0.0010	0.003 0.0051	0.00002 0.000044
NaCl	0.31 0.095 *	0.01 0.085	0.61 0.13 *	0.56 0.19 *	0.30 0.16	0.25 0.21	0.05 0.23	— —

An asterisk indicates a significant difference (more than twice the standard error of the difference) between thresholds. Abbreviations as in Table I. Based on tests in aqueous phase except as otherwise noted.

The comparisons presented in Table II show that the only significant changes in threshold occur when the antennae of the beetles are removed, regardless of the phase in which the stimulus is administered. The high concentrations to which the "refractory" beetles were exposed without obtaining any reaction make it extremely improbable that the beetles ever employ chemoreceptors within the buccal cavity or elsewhere on the body in these experiments.

2. *The specificity of the chemoreceptor groups*

From the data already presented, it appears unlikely that any one appendage bears chemoreceptors sensitive to only one particular modality of stimulus, as suggested by Ritter (1936). As additional evidence on this point, the aqueous phase thresholds of the antennae and palpi to NaCl were determined and the results are included at appropriate places in Table I. It is clear that the receptor area most sensitive to HCl and pentanol (distal tip of the antennae) is also the most sensitive to NaCl, and that the palpi are again of approximately equal sensitivity, as was the case with the alcohol and acid. When the tips of the antennae were removed from animals previously deprived of palpi, the beetles gave no reaction to 1.5 molar NaCl, the highest concentration tested. The question of specialization of various receptors within a single group or area is considered in the discussion.

3. *Structure of the chemoreceptors*

On the basis of evidence thus far presented, it is clear that the chemoreceptors must be located within the distal halves of the distal segments of the antennae, maxillary palpi, and labial palpi. The greater sensitivity of the antennae in both phases suggests that some difference might exist either in the morphological characteristics of the receptors on the antennae and palpi or that the number of receptors might be greater on the antennae. A morphological study of the antennae and palpi was made to discover if any such specializations of structure or number of receptors were apparent. Some of the appendages removed from the operated animals were used for this purpose, and some parts were removed especially for this purpose so that their orientation on the slides could be checked.

The antennae and palpi were placed in a drop of Clarite on a microscope slide and the slide placed in a vacuum chamber. A fifteen-pound vacuum was sustained in the chamber for twenty minutes. This removed any air bubbles formed within the cut end of the material to be observed. After cover slips were in place, the whole-mounts were observed with magnifications up to 1300 ×. Camera lucida drawings of the distal segment, or portions thereof, from the antennae and palpi are shown in Figure 3. All the parts are drawn as they would be seen from the ventral side when the orientation corresponds to that shown in Figure 2. Since, in the living beetles, these appendages are usually waving around vigorously, this manner of orientation may be clearer than trying to orient their surfaces with reference only to the dorsal and ventral aspects of the beetle's body. The magnification scale for *A* and *B* is given in the upper left of the figure and the scale for *C* in the upper right. The entire distal segments of the palpi are shown, and the remainder of the distal antennal segment lacked other receptors.

The key to the presumed sense organs, following the classification of Snodgrass (1935), is as follows: 1-sensilla chaetica; 2-sensilla basiconica; 3-sensilla coeloconica; 4-sensilla placoidea. The sensilla basiconica of the palpi are the same in appearance as those on the antennae, although it was necessary to represent them diagrammatically on this drawing because of their numbers, which are at least several hundred on each palpus. Snodgrass describes the sensilla basiconica as probable chemoreceptors, but little experimental evidence on this point is available. Perhaps the most pertinent evidence is that of Roth and Willis (1951) which strongly indicates that the sensilla basiconica are hygroreceptors in *Tribolium*. It will be noted that the arrangement of receptors in *Laccophilus* makes it practically certain that the sensilla basiconica are the chemoreceptors with which the present experiments are concerned. The only other morphologically distinguishable receptors found in all three of the receptor-bearing areas are the sensilla chaetica and other evidence, as noted below, makes their importance in chemoreception very highly improbable. For example, the sensilla chaetica were found on the other flagellar segments of the antennae and were morphologically and numerically identical on the other segments to those on the distal segment, as well as occupying similar positions on the segments. The same is true for the coeloconic sensillum which is also present on other flagellar segments of the antennae. Hence, if receptors other than the sensilla basiconica were chemoreceptors on the antennae, it would be necessary to assume that similar morphological types of receptors situated in corresponding positions on other antennal receptors were functioning in some entirely different manner. This hypothesis is rejected in favor of the simpler alternative—*i.e.*, that the sensilla basiconica of the antennae are chemoreceptors, and that the other receptor types which are also present elsewhere are not chemoreceptors. The evidence with regard to the palpi is more equivocal. Sensilla chaetica are more abundant (by a factor of four) on the proximal segment of the labial palpi than on the distal segment, and are at least as abundant on the two basal segments of the maxillary

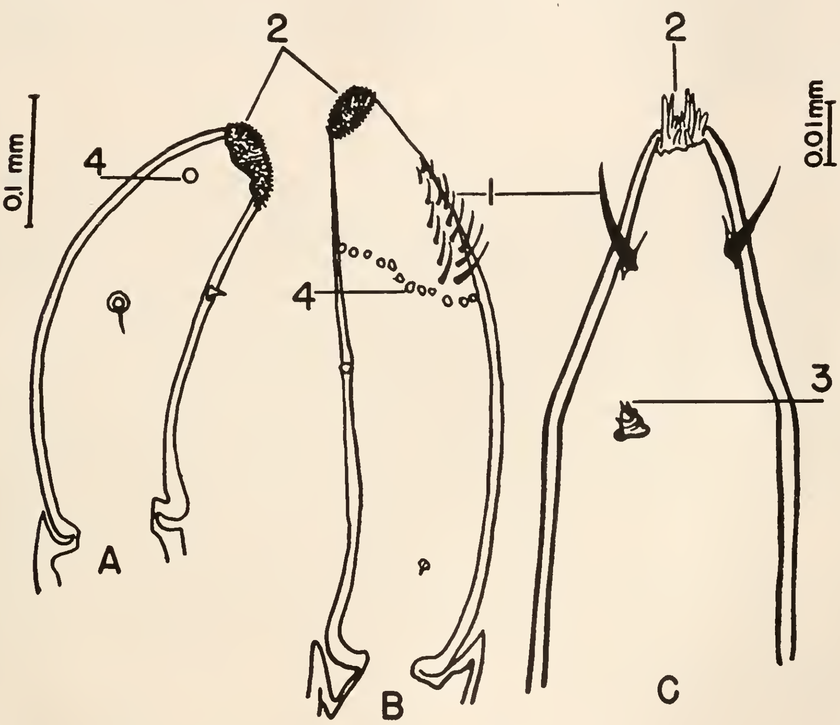

FIGURE 3. Receptors on antennae and palpi. A—distal segment of labial palp; B—distal segment of maxillary palp; C—a portion of the distal segment of an antenna. Key to receptor types and additional explanation in text.

palpi as on the distal segment. Hence, it is unlikely that they are chemoreceptors. The sensilla placoidea are also possible chemoreceptors on the palpi. However, since only one of these organs is present on the distal segment of the labial palpi, it seems unlikely that it alone would be responsible for all the sensitivity of the labial palpi to chemicals. In the case of the maxillary palpi, where the placoid organs circle the distal segment, the placoid organs may be chemoreceptors.

It may be concluded that the sensilla basiconica are the chemoreceptors on the antennae and the receptors chiefly responsible for the sensitivity of this species to gaseous and liquid stimuli. There is also evidence that sensilla basiconica are chemoreceptors on the palpi. It is surprising that the most sensitive region contains the smallest number of sensilla basiconica (tips of antennae) compared to other regions where these receptors are found. The significance of this will be considered in the discussion.

Discussion

Three points merit amplification before drawing final conclusions from this study. The first of these is an assumption which underlies the experiments. This is the assumption that measurements based upon reactions of an entire animal may be used to understand the sensitivity of the animal's receptors. The possibilities for error in making this assumption are probably more obvious than the evidence of its soundness. In the first place, we might expect the assumption to be valid in view of the generalization from comparative psychology that the correlation between structure of the nervous system and behavior of animals becomes clearer as one considers experimental material along phyletic lines from mammals down through the invertebrates (Schneirla, 1952). More pertinent in this particular case is the group of observations already existing on insect chemoreception which shows excellent correlations between known physical and chemical properties of the stimulating compounds and the thresholds for reaction to them (Dethier and Chadwick, 1948). If unknown factors in the nervous systems of the experimental animals were entering into the picture, one would expect that no such clear correlations would be obtained, for they would be obscured by factors within the nervous system modifying the consistent relation between properties of the stimulus applied and the animal's reaction to them.

There is no doubt that electrical measurement of nerve impulses from the chemoreceptors would be the method of choice for use on invertebrates as well as vertebrates, but thus far this has been either technically impossible (Jahn and Wulff, 1950) or limited to detection of narcotic effects which are not specific effects of the stimulating chemicals on chemoreceptors (Hodgson and Roys, unpublished). It is hoped that eventually it will also be technically possible to limit chemoreception studies to a single receptor cell, but in the meantime much of interest may come to light while working toward that end.

In view of the finding that a very small group of antennal receptors mediates reactions to different modalities of chemicals in either gas or liquid phases, it seems logical to next inquire why the receptors on the palpi were not observed to mediate reactions in both phases, since they were sensitive to all three test compounds in the aqueous phase. This discrepancy is resolved by the observation that the thresholds of the palpi are significantly higher than those of the antennae and are, in fact, higher than the concentrations of chemicals which it is possible to accurately administer with the present olfactometer, which reaches its upper limit of concentrations at about one doubling concentration higher than the threshold of these beetles to either pentanol or HCl vapors. Although it is theoretically quite conceivable that some reactions might be obtained by exposing the receptors on the palpi to very concentrated gases, it seems quite certain that in nature the high thresholds of the palpi would prohibit their participation in mediating reactions of the animals to gaseous stimuli.

The difference in threshold obtained when the antennal receptors were tested in gas and liquid phases is, however, of some interest with regard to possible limiting mechanisms in each phase. If the same factor limits the effectiveness of one chemical in stimulating chemoreceptors in two phases, then it should be possible to bring the differences in thresholds into agreement by correcting for the difference in that factor in the two phases. Since some of the information now available on chemoreception suggests that the process is characterized by the establishment of an equilibrium between the concentration of stimulant at the basic site of action and the concentration of stimulant in the phase external to the organism (Dethier and Yost, 1952), it was thought that the limiting factor determining the thresholds in the two phases might be establishment of such an equilibrium. If this were true, the thermodynamic activity of the stimulant, suitably defined, should have the same numerical value in both phases (Ferguson and Pirie, 1948). On the basis of the present experiments, it is possible to make four such comparisons between thermodynamic activities of stimuli at threshold concentrations in aqueous and vapor phases. These values were calculated for pentanol and HCl at threshold concentrations for unoperated beetles, and for beetles with the palpi removed, according to the methods of Ferguson and Pirie (1948), and Brink and Posternak (1948). Since the thermodynamic activities at threshold were not even of the same order of magnitude when data from similarly treated beetles in the two phases were compared, this approach will not be elaborated upon here.

The essential point is that either chemoreception does not depend upon the establishment of an equilibrium in the two phases or else some behavioral difference in the testing methods used in the two phases masks the identity of the fundamental mechanisms. In this connection it is interesting to note that Dethier and Yost (1952) found that alcohols of intermediate chain length when stimulating blowflies as gases obeyed the law of equal effect at equal thermodynamic activity; however, this principle did not hold when the tarsal receptors of the blowfly are stimulated by alcohols, although here, too, the behavioral criteria might be involved in the discrepancy.

Finally, the bearing that these findings have on the concepts of olfaction, gustation, and sensory modalities should be mentioned. It must be conceded that classifying stimuli as odor-substances or taste-substances is simply making a difficult problem more obscure and is a practice which should be abandoned. In view of the present results, the advisability of making a distinction between olfactory and gustatory receptors on the basis of the physical state of the stimulus to which they are sensitive could be similarly misleading, since a very restricted group of receptors of the same morphological type can mediate reactions to both gaseous and liquid stimuli. It is remarkable that the smallest number of sensilla basiconica is on the region most sensitive to chemicals and a much larger number on the palpi which are relatively insensitive. This indicates that the sensitivity of the antennal receptors is the result of some specialization of the receptor cells themselves rather than simply the result of a great many receptors summing nerve impulses as is sometimes advanced by way of an explanation for the greater sensitivity of olfaction as compared with gustation (Moncrieff, 1944).

There is no evidence in these data for the existence of topographically separated receptors sensitive only to particular modalities of stimuli as was concluded by Ritter (1936). It is possible that the apparent specificity of the receptors on the labial palpi for acids resulted from having the concentrations of salt and non-acid stimuli below threshold, while the acid happened to be above the threshold of the receptors on the labial palpi. This particular point is more relevant to understanding chemoreception in insects than to concepts about the chemical senses in general, since it merely indicates that the sense organs of insects have not evolved the same types of specializations as those found in mammals. Of course it cannot be assumed that different sensilla within a particular group necessarily have identical physiological functions and thresholds, any more than it could be assumed, for example, that different sensilla with cuticle of similar thickness have the same permeability properties (Richards, 1952). Such a condition would be, however, quite different from the grouping of specialized receptors suggested by Ritter (1936). This leaves the problem of the existence of modalities among chemicals stimulating for insects, and especially the basis of their discrimination by the animal, in a very unsatisfactory state. If different classes of chemicals do not activate different receptors, how are they discriminated as classes of stimuli? One wonders how many such classes of compounds might be found in a systematic experimental survey not organized around the classic modalities of taste stimuli for humans. Much additional evidence on this point is needed, but the suggestion by Frings (1946) that the modalities represent only points in a continuous spectrum of taste sensations related to some surface-active property of the chemicals might offer a less anthropomorphic approach for experimentation than some of those used in the past.

SUMMARY

1. Quantitatively controlled stimuli were administered to populations of an amphibious beetle, *Laccophilus maculosus* Germar, to determine whether the same chemoreceptors are sensitive to gaseous and liquid stimuli, and to discover qualitative or quantitative specializations in the function of different receptor groups.

2. Sensilla basiconica on the tips of the antennae are the principal chemoreceptors for both gaseous and liquid stimuli. The lower threshold of antennal receptors, relative to receptors elsewhere on the animals, is not due to a larger number of sensilla on the antennae and indicates an inherent specialization of the receptors themselves.

3. Morphological and experimental evidence strongly indicates that the sensilla basiconica on the tips of the maxillary and labial palpi also function as chemoreceptors for stimuli in solution, although their thresholds are higher than those of antennal receptors. The concentration of gases could not be raised to a level adequate for stimulation of receptors on the palpi and they must play little or no part in mediating reactions of the animals in air.

4. HCl, 1-pentanol, and NaCl all stimulated receptor areas on the tips of the antennae and palpi, and no evidence was found for specialization of any morphologically or topographically distinguishable receptor groups sensitive to only a particular

modality of stimulus. Chemoreceptors were not found on parts of the body other than the antennae and palpi.

5. The findings are discussed with reference to possible identity of fundamental mechanisms limiting the effectiveness of chemical stimuli in the two physical states, and general concepts of olfaction and gustation.

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THE PERMEABILITY OF THE ERYTHROCYTE-LIKE CELLS OF PHASCOLOSOMA GOULDI[1]

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The coelomic fluid of the annelid worm *Phascolosoma gouldi* contains cells of unknown function which at least superficially resemble vertebrate erythrocytes (Cuénot, 1891; Dawson, 1933). Like the latter, they have two special properties which permit the use of Jacobs' hemolysis method (1934) to determine their permeability constants. First, they lose their red pigment, hemerythrin, on swelling osmotically to a certain critical volume; the accompanying optical changes are as striking as those seen in the hemolysis of vertebrate erythrocytes. Second, their discoidal shape enables one to assume that the cell surface area does not change significantly during moderate osmotic swelling.

This paper reports the application of Jacobs' method to Phascolosoma "erythrocytes." Measurements have been made of (1) normal cell volume, (2) "hemolytic" volume, (3) cell surface area, and (4) "hemolysis" times in water and various solutions isosmotic with sea water. From these data, permeability constants have been calculated and compared with those of certain representative cells for which quantitative data exist.

MATERIAL AND METHODS

To make stock suspensions of Phascolosoma erythrocytes, coelomic fluid from ten to twenty worms was diluted to about 100 cc. with filtered sea water, which Chapeau (1928) has reported to be isotonic. Filtration through a loose plug of absorbent cotton removed the large eggs and the clot which usually formed. Repeated gentle centrifugation served to separate the erythrocytes from the much smaller sperm cells which remained suspended. The packed cells from the final centrifugation were used as the stock suspension.

Normal cell volume was determined from 8 hematocrits combined with 5 cell counts of the same suspension. The average cell volume was 1050 μ^3.

It was obviously impossible to measure accurately the hemolytic volume during hemolysis. Instead, it was estimated from microscopic measurements of the diameters of intact spherical cells remaining in the dilute sea water which previously had been found to produce 50% hemolysis. The mean diameter of fifty such cells was 14.5 μ with a standard deviation of 1.8 μ; thus the average hemolytic volume was 1610 μ^3, and the average surface area was 660 μ^2, assuming constancy during swelling. The swollen cell volume, calculated for the 60% sea water used above by the van't Hoff law from the normal cell volume, exceeds the measured hemolytic volume by an amount corresponding to a non-solvent space of 20% of the cell.

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This "dead-space" is higher than that of 7–14% for Arbacia eggs found by McCutcheon *et al.* (1931) and lower than that of about 30% reported by Ponder (1948) for mammalian erythrocytes. Although it is recognized that there may conceivably be a selective effect of this procedure for estimating hemolytic volume, the method has been used because of convenience. Its use is supported by the fact that the non-osmotic volume so estimated is in reasonable agreement with the high hemerythrin content of these cells (Florkin, 1933).

The non-electrolyte solutions were made one molar, which is osmotically roughly equivalent to Woods Hole sea water, and were lightly buffered to approximately the pH of sea water (by addition of 5% by volume of 0.33 M Na_2HPO_4 which had been brought to pH 7.8 with concentrated HCl). As a result, the pH remained essentially constant during hemolysis of added cells. One-half molar solutions of ammonium salts of fatty acids were prepared by titrating a small quantity of the proper undiluted fatty acid with $M/2$ NH_4OH to a pH such that equal amounts of ammonia and fatty acid were present in the solutions (see, in this connection, Stewart, 1934). To measure a hemolysis time in one of these solutions, 0.05 cc. of the stock suspension of cells was mixed with 10 cc. of the solution; the time of 50% hemolysis was determined by the "slit" method of Jacobs *et al.* (1949). Control experiments showed that none of the substances discussed below were hemolytic *per se*.

Results and Discussion

The observed hemolysis times are listed in the second column of Table I. In the third column are listed the corresponding permeability constants, calculated by the method of Jacobs (1934) from hemolysis times, hemolytic volume, and normal cell volume and surface area. For comparison the permeability constants of unfertilized Arbacia eggs (Jacobs and Stewart, 1932), those of cells of *Chara ceratophylla* (Collander and Bärlund, 1933) and those of beef erythrocytes (Jacobs, 1952) are also given. To fill out the table, the permeability constants of the beef erythrocyte for amides were determined by Jacobs' hemolysis method. They are in good agreement with the experiments of Höber and Ørskov (1933). Following Collander and Bärlund (1933), for solutes P is the number of mols crossing one $cm.^2$ of membrane in one hour with a concentration difference of one mol per cc., while P_w is the cc. of water crossing one $cm.^2$ of membrane with an osmotic pressure difference of one atmosphere.

Two general qualitative principles have emerged from the past studies of permeability: (1) small molecules diffuse into living cells faster than larger ones, (2) lipoid-soluble molecules diffuse into living cells faster than those which are not lipophilic (Höber, 1945). Phascolosoma erythrocytes follow the above rules rather well, as is indicated by their permeability constants in Table I. For example, permeability to alkyl amides increases with increasing hydrocarbon chain length. This probably results from a stronger influence of lipoid solubility than of molecular volume on diffusibility of the molecules. Probably for the same reason esterification of glycerol with acetic acid increases the rate of entrance. On the other hand, in the series of glycols where molecular volume increases without much change in lipoid solubility, the expected decrease in the ease of penetration is seen. Further agreement with the properties of other cells is shown by the increased permeability to 1,3 propandiol versus glycerol, as well as higher permeability to 1,2 propandiol than to its 1,3 isomer.

The results in Table I show that Phascolosoma coelomic cells resemble the other nucleated cells and that they do not possess the special high permeability to both water and urea of beef erythrocytes. In this respect the cells here studied do resemble the erythrocytes of the elasmobranchs, amphibia and reptiles, although only qualitative comparisons can be made with the data of Jacobs *et al.* (1950). In addition, in the beef erythrocyte the effect of lipoid solubility on ascending the homologous series of amides is not apparent from the first member of the series as it is with Arbacia eggs and Phascolosoma erythrocytes.

TABLE I

Permeability constants of selected cells for various substances

Substance	Time in seconds for 50% hemolysis of Phascolosoma "erythrocytes" at 25° C.	$P \times 10^4$ (cm. × $hr.^{-1}$)			
	Solute 1 Molar	Phascolosoma	Arbacia eggs	Chara leaf cells	Beef red cells
Formamide	158	59		770†	1760
Acetamide	124	95	345‡	530†	1700
Propionamide	73	374	850‡	1300†	685
Butyramide	53	2460	2200‡	1700†	980
Ethylene Glycol	185	44	240*	430†	58*
Diethylene Glycol	281	21	150*		27*
Triethylene Glycol	637	7.1			12*
Tetraethylene Glycol	1573	2.5			
Urea	552	9.6		40†	6500*
Thiourea	423	12.1		77†	7*
Glycerol	more than 14,400	less than 0.025	3‡	7†	0.7*
Monacetin	1137	3.7		160†	41*
Diacetin	248	26.8		800†	375*
1,3 Propandiol	186	44	260*		36*
1,2 Propandiol	145	69	460*	870†	118*
		P_w ($cm.^4 \times hr.^{-1} \times mol.^{-1}$)			
Water	49.2	72	22§ (exosmosis) 18|| (endosmosis)		400*

* Jacobs (1952); † Collander and Bärlund (1933); ‡ Jacobs and Stewart (1932) § Jacobs (1932); || Lucké, Hartline and McCutcheon (1931).

Experiments with electrolytes throw further light on this question of the general classification of Phascolosoma erythrocytes with respect to their permeability. Their lysis is quite rapid in *M*/2 ammonium acetate, but does not occur in four hours in either *M*/2 ammonium chloride or *M*/2 sodium acetate. The cells therefore appear to be permeable to both ammonia and acetic acid and evidently much less permeable to either sodium ions or chloride ions (Jacobs, 1927). In this respect, therefore, they resemble typical plant and animal cells rather than the vertebrate erythrocyte, in which a special free permeability to anions facilitates the transport of carbon dioxide.

Permeability constants calculated from the hemolysis times in isosmotic solutions of ammonium salts of fatty acids do not have the same significance as those already mentioned because of the complex manner in which these salts enter cells (Jacobs, 1927). However, the hemolysis times shown in Table II, like the results obtained with other cells (Jacobs, 1927; Bouillenne, 1930; Stewart, 1934), indicate that increasing lipoid solubility of the fatty acid goes hand in hand with an increase in the rate of entrance of the salt. It should be noted that in the series: acetate, propionate, butyrate, and valerate, the lysis times are entirely comparable because the pK values of the four acids in question are nearly the same.

Table II

50% hemolysis times in M/2 solutions of ammonium salts of fatty acids

Ammonium salt	Time in seconds for 50% hemolysis of Phascolosoma erythrocytes at 25° C.
Formate	1,145
Acetate	388
Propionate	158
Butyrate	93
Valerate	52

The hemerythrin-containing cells of Phascolosoma coelomic fluid resemble the erythrocytes of the lower vertebrates morphologically. Moreover, they contain a high concentration of a red pigment which combines loosely and reversibly with oxygen (Cuénot, 1891), and which they lose by a process analogous to hemolysis of vertebrate erythrocytes. Yet in most instances the present results show that substances enter the Phascolosoma cells more nearly the way they enter Chara cells and Arbacia eggs; in particular, Phascolosoma erythrocytes lack the special free permeability to urea, water, and anions characteristic of the beef erythrocyte.

The author wishes to express his gratitude to Prof. M. H. Jacobs for his help during the course of the work, and to Dr. T. F. Anderson for his aid in preparing the manuscript.

Summary

By virtue of two properties, lysis on reaching a critical volume, and a discoidal shape, the hemerythrin-containing corpuscles of the coelomic fluid of *Phascolosoma gouldi* have proven to be excellent material for the quantitative estimation of permeability constants. The constants evaluated by the method of Jacobs from times of lysis show a general similarity between these cells and unfertilized Arbacia eggs in respect to permeability to water and a series of non-electrolytes. The Phascolosoma cells also resemble Arbacia eggs in their low permeability to both cations and anions.

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NUCLEO-CYTOPLASMIC INTERACTION DURING CONJUGATION IN TETRAHYMENA[1]

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Studies on the ciliated Protozoa, particularly *Paramecium aurelia* (Sonneborn, 1950b, 1951), have provided much information on the roles of the nucleus and the cytoplasm in cellular differentiation. One important observation in these studies (Sonneborn, 1947) is that the cytoplasm may control the kind of macronucleus developed in a cell; specifically, the cytoplasm may determine whether a new macronucleus will differentiate so as to control one or the other of the possible mating types. It has been suggested (Nanney, 1953) that the cytoplasm in these cells has been determined by the kind of macronucleus previously occupying the cell. This does not detract from the importance of the cytoplasm in cellular heredity, but emphasizes the importance of nucleo-cytoplasmic *interactions*.

Related to this problem of what determines the *kind* of macronucleus to develop is the problem of what determines *whether* a particular nucleus will differentiate into a macronucleus. In many ciliates the fertilization nucleus produced at nuclear reorganization divides twice to produce four presumably identical nuclei: two of these differentiate as macronuclei and two as micronuclei. Long ago Maupas (1889) suggested that this difference in the development of nuclei was due to localized differences in the cytoplasm surrounding the nuclei at a critical time in their development. Maupas based this suggestion on observations made on a group of ciliates, including particularly *Colpidium, Leucophrys* and *Glaucoma.* He observed that in these organisms the fertilization nucleus divided twice and that the spindles for the second post-zygotic division were oriented in such a fashion that two of the four division products in each cell were placed at the extreme anterior end of the cell and two were placed at the extreme posterior end of the cell. Those which were placed at the anterior end of the cell were observed to enlarge and become the new macronuclei while those at the posterior end remained small and became the new micronuclei. Here was a clear visible correlation between the location of a nucleus in the cytoplasm and its subsequent development. Although this correlation strongly suggested cytoplasmic control of nuclear development, other interpretations were possible and were not excluded.

A number of observations similar to those of Maupas have been made on a variety of organisms since Maupas' time and recently Sonneborn (1951) has directed attention to the nuclear events at conjugation in *Paramecium* where several additional instances of apparent cytoplasmic control of nuclear activity are

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found. The present study is presented as an extension of his observations to another organism, with certain experimental data bearing on his conclusions.

MATERIALS AND METHODS

Strains of *Tetrahymena* were recently obtained (Elliott and Nanney, 1952) which permitted further analysis of some of these problems. These strains, designated as the AA strains, undergo conjugation regularly. The entire process has been analyzed both to gain insight into the factors controlling nuclear development and to provide a firm basis for subsequent genetic studies. The eight strains studied were all collected in the vicinity of Ann Arbor, Michigan and in each of them any isolated cell will give rise to a culture in which conjugation (selfing) occurs. However, no conjugants have been found which give rise to viable progeny. More recently (Elliott and Gruchy, 1952), other strains of *Tetrahymena* were collected near Woods Hole, Massachusetts, and in these strains—designated as the WH strains—Elliott has demonstrated that mating types exist and that viable progeny are produced. The details of conjugation in the WH strains resemble closely those in the AA strains with the exception of a few modifications related to the occurrence of mating types and a high frequency of spontaneous anomalies in the cytogenetic processes. These differences and their significance will be discussed in a later paper.

The selfing strains are designated as AA 1–8, and have been assigned to the genus *Tetrahymena* by Corliss and Furgason (in Elliott and Nanney, 1952). They show certain differences from the strains of *T. pyriformis* (=*geleii*) extensively studied and are, hence, described simply as *T. sp.* One of the principal differences between these strains and the long-maintained laboratory cultures of *T. pyriformis* lies in the fact that the latter are lacking in micronuclei. *T. sp.* has, as a rule, a single micronucleus, but occasional cells with as many as four micronuclei have been observed.

Most of these strains, perhaps all, can be grown on a defined medium (Elliott and Nanney, 1952), but in the present study all the cells were maintained in bacterized cultures. The medium was prepared by boiling 1½ grams of Cerophyl in a liter of distilled water, filtering and autoclaving. The day before the medium was to be used, it was inoculated with *Aerobacter aerogenes*. The general culture methods followed closely those described for *Paramecium* by Sonneborn (1950a).

Conjugation occurs regularly in all the stocks soon after the nutrient in the medium is exhausted. It has not been possible even after many serial isolations to derive cultures differing in mating type; any isolation gives rise to a clone within which nearly 100% conjugation can occur. No evidence was found for autogamy or any other process of nuclear reorganization which might account for diversities within a culture; hence, it seems reasonable to assume that the cells which conjugate are genetically alike. The question of whether the cells are of different mating types will be discussed in a later paper, but at the present time no evidence is available for any differences between the cells that conjugate.

Under all conditions thus far tried, the exconjugants die—usually without separating. The cultures are perpetuated from the individuals which have failed to conjugate. Attempts to obtain viable conjugants by growing cells from different sources in the same container and isolating conjugants have proved unsuccessful.

Since conjugation occurs within a single culture, it is difficult to control its initiation. Pairs are formed over a period of several hours and samples removed at any one time contain pairs in many stages of conjugation. The sequence of stages must, therefore, be inferred rather than directly demonstrated. Similarly the length of time necessary for the completion of the various stages cannot be determined readily.

In preparation for cytological studies, pairs were killed and fixed in hot Schaudinn's solution. Various staining techniques were used: these include the borax-carmine method of Dippell (in Sonneborn, 1950a), Dippell and Chao's (in Sonneborn, 1950a) modification of the De Lamater stain and the Giemsa method described by Preer (1950).

The figures representing the sequence of stages are camera lucida drawings of stained pairs. No attempt has been made to simulate the structural details of the nuclei or to indicate the precise number or size of the chromosomes. The chromosomes are small, numerous and difficult to count or draw, though this should be possible eventually. Characteristic changes may be noted in the staining properties of the chromosomes and these will be described later.

DESCRIPTIVE

1. *The normal pattern of nuclear behavior*

The normal pattern of nuclear behavior during conjugation closely resembles that reported by Maupas (1889) for *Leucophrys patula*. Recent systematic revisions (Furgason, 1940; Corliss, 1952) indicate that *Leucophrys patula* is more correctly termed *Tetrahymena patula*, a species closely related to *T. sp.;* hence, this similarity in nuclear behavior is not surprising. In spite of the similarity in the cytogenetic details reported by Maupas and those reported below, it appears advisable to present briefly the normal sequence of events as a basis for comparison with the anomalies to be presented subsequently.

Conjugating cells attach at their oral surfaces (near the anterior ends of the cells) with a "face to face" orientation. Preliminary clumping reactions and non-specific attachments have not been observed, but it is possible that these occur. At the time the cells first become attached, the single micronucleus is found near the macronucleus but may be anterior, posterior or lateral to it. Shortly after the initiation of conjugation the micronucleus moves into a region just anterior to the macronucleus (Fig. 1A), enlarges and begins to elongate into the typical "crescent" stage of the first pre-zygotic division (Fig. 1B). This crescent shortens in the later stages of this division (Fig. 1C) and the chromosomes become clearly visible. The first division is completed and the two daughter nuclei enter immediately into the second pre-zygotic division (Fig. 1D) still anterior to the macronucleus. During this division the chromosomes are much less prominent than during the previous division. Extrapolating from information on other ciliates whose genetics are well known (see Sonneborn, 1947), it appears probable that these first two divisions are the meiotic divisions and that the resulting four nuclei are haploid.

At the end of the second pre-zygotic division the four nuclei in each cell continue to migrate anteriorly until one of the nuclei comes in contact with the membrane between the cells and appears to attach to it. More specifically, the nuclei attach on the right side of the cell. The attachment of nuclei on opposite sides of the contact surfaces is usually, but not always, synchronous. Following the attachment of one of the nuclei, the remaining nuclei in the cell begin to move posteriorly (Fig. 1E *et seq.*), eventually to disintegrate. These "relic" nuclei may persist for a variable length of time, but have never been observed to undergo any further divisions.

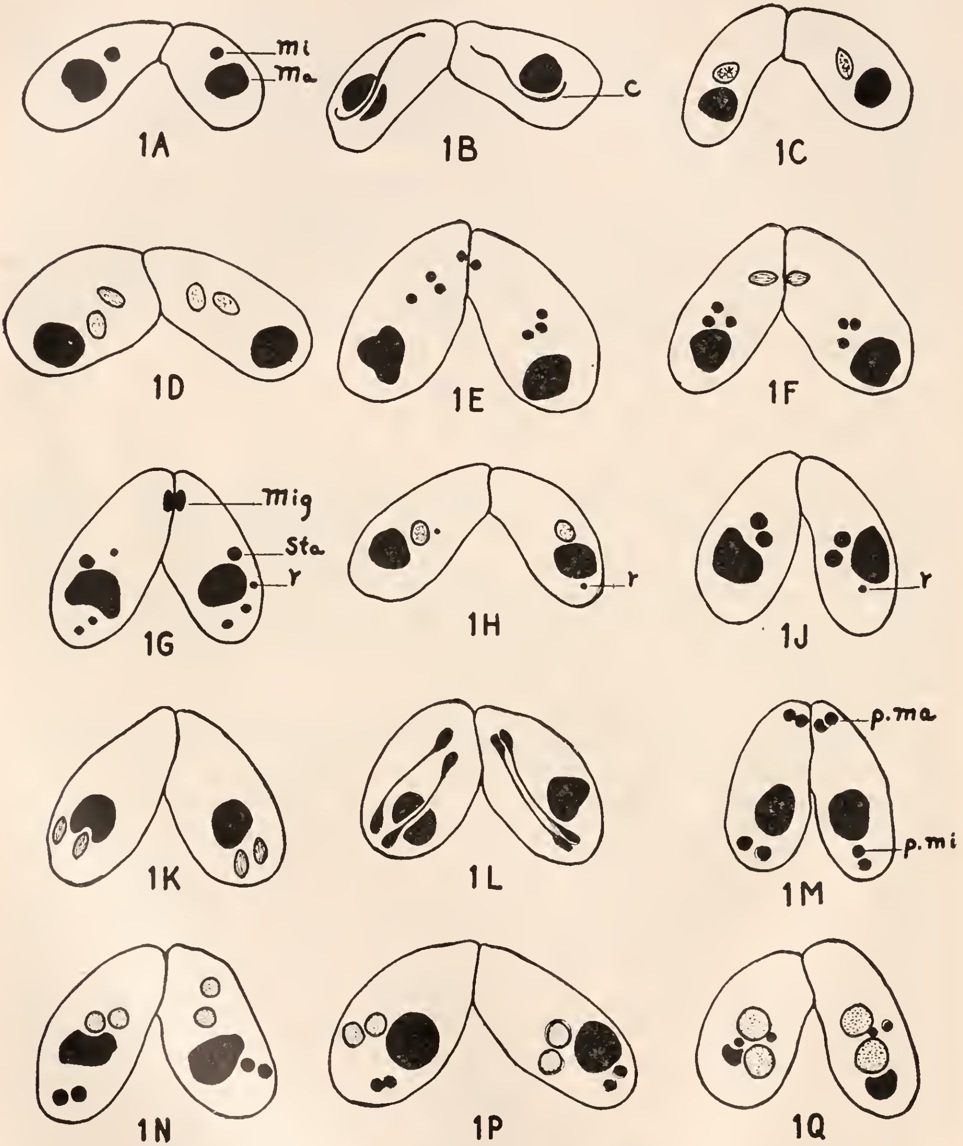

FIGURE 1. The normal sequence of nuclear changes at conjugation in *Tetrahymena sp.; mi* = micronucleus; *ma* = macronucleus; *c* = crescent; *r* = relic nuclei; *mig* = migratory nucleus; *sta* = stationary nucleus; *s* = syncaryon; *p. ma* = presumptive macronucleus; *p. mi* = presumptive micronucleus. See explanation in text.

The nucleus situated at the membrane enlarges and undergoes a third prezygotic division while still attached (Fig. 1F). Since the previous divisions are probably the meiotic divisions, this may be assumed to be an equational division. One of the nuclei produced by this division (the migratory nucleus) remains at its original position; the other nucleus (the stationary nucleus) moves to a region just anterior to the macronucleus (Fig. 1G) and may be distinguished from the relic nuclei by its larger size and more spherical shape. By this time these other haploid nuclei are often pycnotic and in some instances have already disintegrated.

The migratory nucleus is observed to flatten considerably (Fig. 1G) and then to round up and protrude slightly into the other cell. Eventually the migratory nuclei from the two pair members are exchanged. The fertilization nucleus is formed by the fusion, just anterior to the macronucleus, of the migratory nucleus from one cell with the stationary nucleus from the other cell. This fertilization nucleus immediately prepares to divide (Fig. 1H). Since the fertilization nucleus is formed by the union of presumably identical nuclei in the two members of a pair, it is necessary that the two exconjugants of a single pair will be alike in their genetic constitution.

Immediately following fertilization the syncaryon undergoes its first post-zygotic division while still anterior to the macronucleus. The two large conspicuous daughter nuclei (Fig. 1J) migrate posteriorly and the second and last post-zygotic nuclear division is initiated posterior to the macronucleus with the spindles oriented longitudinally in the cell (Fig. 1K). These spindles elongate until they extend nearly the entire length of the cell (Fig. 1L), and at the end of this division two nuclei are left at the extreme anterior end of the cell and two at the extreme posterior end of the cell (Fig. 1M). Those at the anterior end, the presumptive macronuclear anlagen, begin to move toward the posterior end, to enlarge and stain less intensely than before (Fig. 1N and 1P). The nuclei at the posterior end, on the other hand, remain small and become the new micronuclei.

At about this time the original macronucleus shows the first evidence of change. It loses its irregular outline, becomes spherical and deeply staining (Fig. 1P). Eventually it becomes smaller and is lost (Figs. 1Q–2B), although not invariably at the same time in both conjugants.

The pair members usually do not separate, though they may be forcibly separated during the latest stages and a few pairs separate spontaneously. Often the conjugating cells coalesce (Fig. 2C), become spherical and vacuolated, and finally lyse. The conjugants which separate likewise do not survive.

2. *Conjugation involving three cells*

Maupas (1889) reported having seen triple formations many times in *Leucophrys patula*, as well as in other ciliates, but gave no further information on either the mode of attachment or on the cytogenetic details. Triples are regularly seen in the AA cultures and may involve a small percentage of the observed conjugants. In all cases thus far observed the triples are formed by the symmetrical union of cells at the oral surfaces. No attachments at other points have been observed. In the WH *Tetrahymena* strains a different kind of triple has been found. These triples are due to the simultaneous union of two single animals to the two oral surfaces of a "double" animal. Such triples are not to be confused with the tripolar triples reported here. A third type of triple is known in many ciliates and occurs when a third cell becomes attached in any of a variety of positions to one member of a conjugating pair. In *Paramecium bursaria,* Chen (1946) has shown that this third mate undergoes autogamy and neither receives a pronucleus from nor contributes a pronucleus to either of the other cells. Weisz (1950) reports triple formations in *Blepharisma,* some of which appear to be similar to those studied by Chen, but others of which may be of the tripolar sort described below

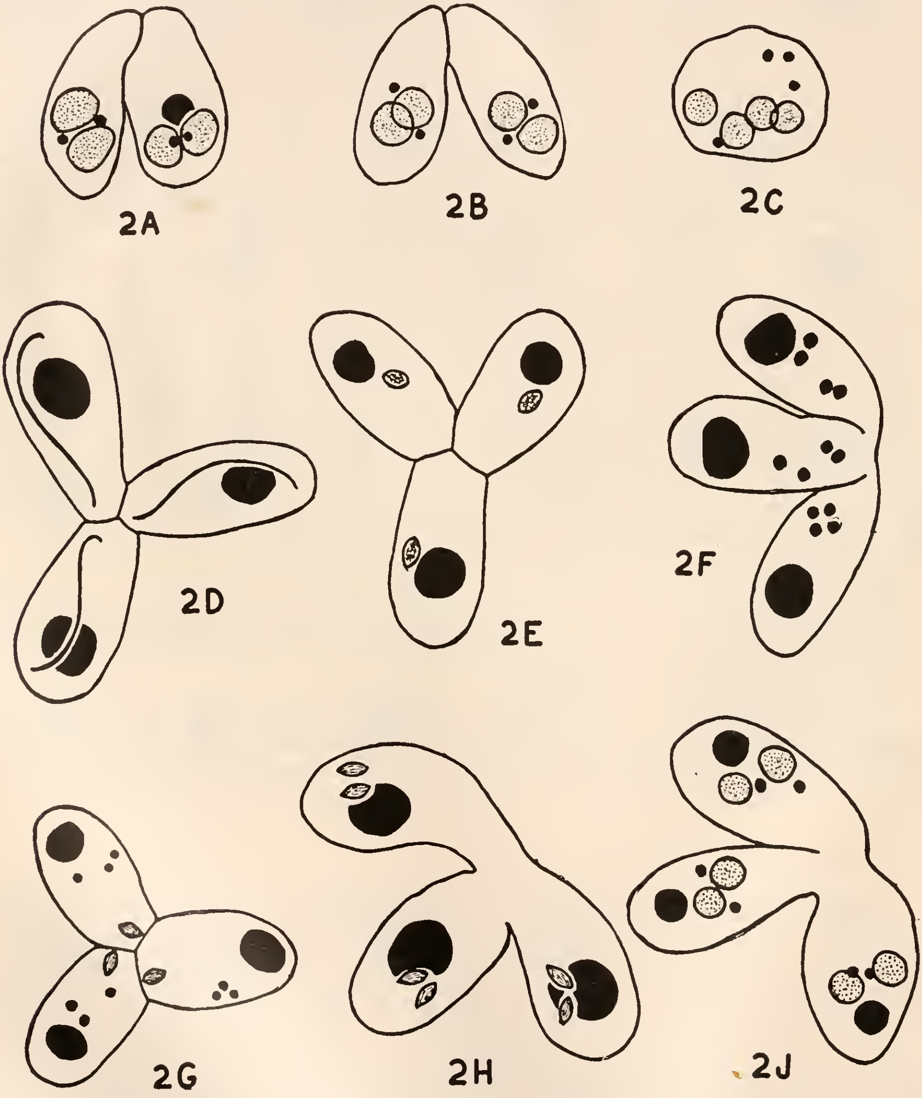

FIGURE 2. 2A–2C, terminal stages in normal conjugation. 2D–2J, conjugation in triples. See explanation in text.

for *Tetrahymena*. Since nuclear studies on the *Blepharisma* strains were not conclusive, this interpretation must remain tentative.

The pattern of nuclear behavior in the AA triples is precisely identical with that in pairs; all three cells appear to undergo a normal reorganization with the normal synchrony. Figure 2D shows a typical crescent stage; Figure 2E shows a later stage in the first pre-zygotic division. Figure 2F shows the four nuclei in each cell produced by the second pre-zygotic division. Figure 2G shows the spindles for the third pre-zygotic division. It will be noted that the dividing nuclei are all attached on what appears to be the left side of the cell (actually the right side, judging from the nuclear orientation in pairs where right and left are readily determined). It appears likely that in this case the migratory nucleus passes through the membrane to which it is attached and, hence, that each cell contributes a migratory nucleus to one cell and receives a migratory nucleus from the other cell. This is, therefore, in all probability a true tripolar fertilization and should yield different genetic results than normal conjugants. Specifically, tripolar fertilization could yield three genetically diverse cells after conjugation under some circumstances. Figure 2H shows the spindles for the second post-zygotic nuclear division and Figure 2J illustrates the stage after the new macronuclei have differentiated, but before the old macronucleus has completely disappeared.

3. *Conjugation in cells with multiple micronuclei*

Within mass cultures of several of the stocks occasional cells have been observed with multiple micronuclei. In one isolation line nearly all the cells showed two micronuclei at the time they were first stained. Subsequently the frequency of bi-micronucleate cells decreased and the culture returned to the uni-micronucleate condition. During the period when the culture possessed many bi-micronucleate cells, pairs were stained and studied.

Multiple micronuclei appear in no way to affect the behavior of the individual nuclei or to alter the consequences of conjugation. All the micronuclei originally present undergo the first and second pre-zygotic divisions. Figure 3A shows a pair in which both conjugants have two micronuclei and in which all the micronuclei are in the crescent stage. Figure 3B shows a pair in which one cell has one micronucleus and the other has two. Figures 3C–3E show the later stages in the first pre-zygotic division in conjugants with a variety of nuclear constitutions. Figure 3F shows the beginning of the second pre-zygotic division in a pair which originally consisted of a uni-micronucleate and a bi-micronucleate member. The chromatin in this stage appears as a faintly staining network, strikingly different from the chromatin in Figures 3C–3E, in which distinct chromosomes are readily seen. Figure 3G shows a pair similar to that in 3F, but after the second pre-zygotic division; eight nuclei are seen in one cell and four in the other. Figure 3H shows a pair beginning the third pre-zygotic nuclear division; the one dividing nucleus and the seven relic nuclei in each cell indicate that both cells were originally bi-micronucleate. It is observed that only one micronucleus ever undergoes the third pre-zygotic division, regardless of whether a cell contains four, eight or twelve (when the cell was originally tri-micronucleate). Following fertilization and the disintegration of the relic nuclei, no differences can be ascertained between cells which were originally multi-micronucleate and those that were originally uni-micronucleate.

The number of micronuclei present in a cell appears to have no influence on either the probability of mating or on the kinds of matings observed. The frequency of multi-micronucleate cells in conjugation is not significantly different from the frequency of such cells in the same culture which are not conjugating at

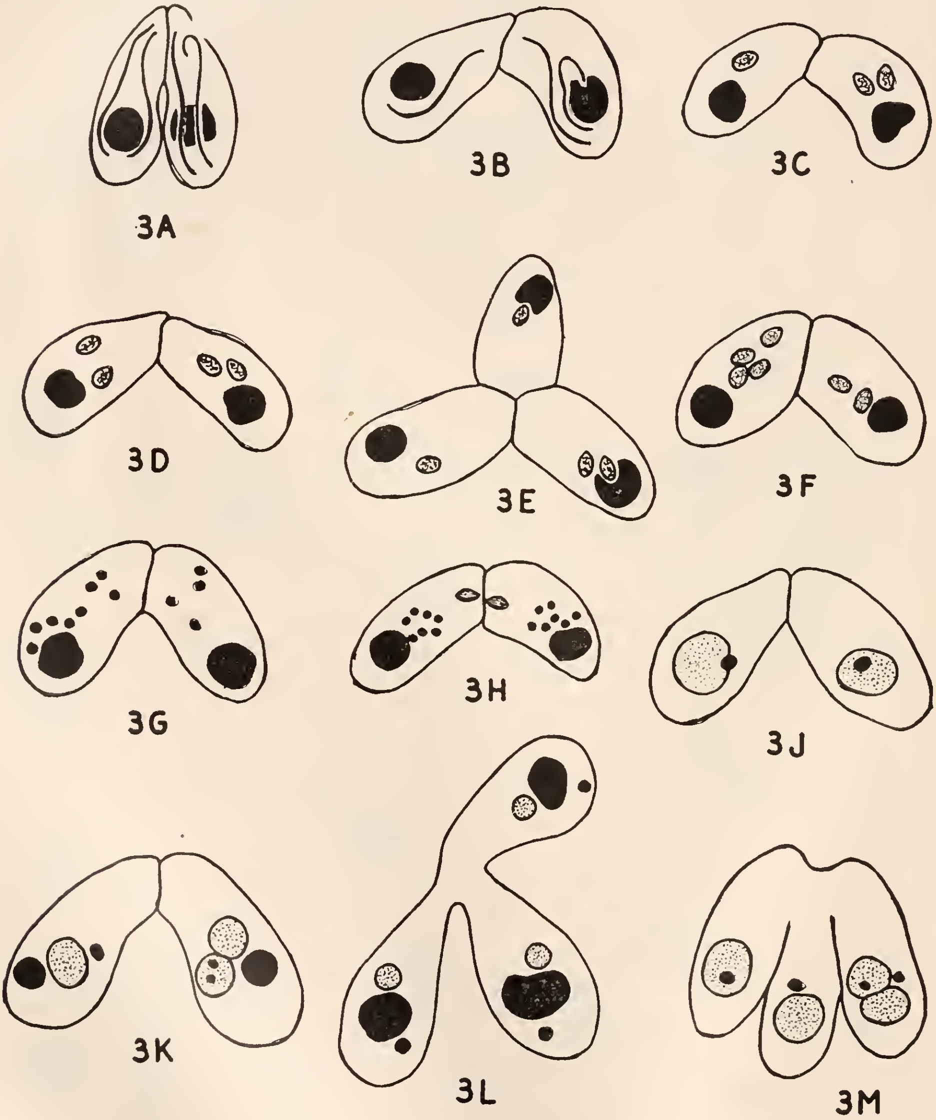

FIGURE 3. 3A–3H, conjugation in cells with multiple micronuclei. 3J–3M, a spontaneous anomaly. See explanation in text.

a particular time. The distribution of pair types (uni-micronucleate × uni-micronucleate, uni-micronucleate × bi-micronucleate, etc.) is not significantly different from the distribution expected by chance alone.

4. *A spontaneous anomaly*

Only one spontaneously occurring anomaly has been observed in the AA cultures, and this occurred in about 7% of the conjugants (45 of 608 on one slide) in a single stock (AA-1) the first time conjugation was observed; it has not been encountered since. Unfortunately, the culture in which the anomaly was found was in the later stages of conjugation, and it was not possible to determine the manner in which the abnormality developed. This anomaly is characterized by the presence at the end of conjugation of only one new macronucleus and only one new micronucleus instead of the usual two of each kind (Fig. 3J–3M). These new nuclei, both macro- and micronuclei, are clearly larger than those in normal cells. Rough measurements show a difference in volume of a factor of two compared with the nuclei in comparable stages of normal cells.

An examination of prepared slides was undertaken to determine whether pair members tended to resemble each other in respect to the number of new nuclei produced. On a particular slide 270 pairs were found, in which both conjugants were normal; 23 pairs were found with one abnormal member and 11 pairs were observed to have two abnormal members. On simple probability considerations the expected classes are 260, 42 and 2. It seems probable, therefore, that pair members tend to be alike. The significance of this observation is not clear.

Experimental

The fact that differences in the behavior of different nuclei present in the cell at the same time are correlated with the regular localization of the nuclei in specific cytoplasmic regions is sufficient to suggest that nuclear behavior is to some extent controlled by local differences in the cytoplasm. This fact alone, however, may not be considered critical evidence for such a cytoplasmic role. It is conceivable that the nuclei are self-determined to behave as they do regardless of where they are located. If, however, nuclei could be transferred from one cytoplasmic locality to another and if this relocation could be demonstrated to result in altered nuclear behavior or nuclear differentiation, the hypothesis of cytoplasmic control could be considered firmly established.

The simplest method for relocating nuclei in the cytoplasm appeared to be centrifuging. Cultures in conjugation were centrifuged in an International Clinical Centrifuge at full speed (about 5000 g) for various lengths of time; they were allowed to recover for from one to 24 hours and were then fixed and stained. Some cultures were centrifuged for ten minutes; other cultures were centrifuged for ten minutes, allowed to recover for fifteen minutes and were again centrifuged. In some instances three periods of centrifugation were used.

Cultures stained immediately after centrifugation showed that the nuclei were indeed relocated in the cytoplasm, and slides prepared at various intervals after the cells had recovered showed that alterations in nuclear behavior had been accomplished. These alterations may be described as follows.

1. *Simple mate-to-mate transfer*

The commonest abnormality observed was the transfer of part of the nuclear equipment from one cell into its mate. Observations suggest that this transfer may occur during any stage of conjugation. Figure 4A shows a pair in which the entire micronuclear material of one cell was transferred to the other cell at some stage prior to fertilization and probably prior to the attachment of a nucleus to the membranes separating the cells. One cell contains no micronuclei; the other contains seven

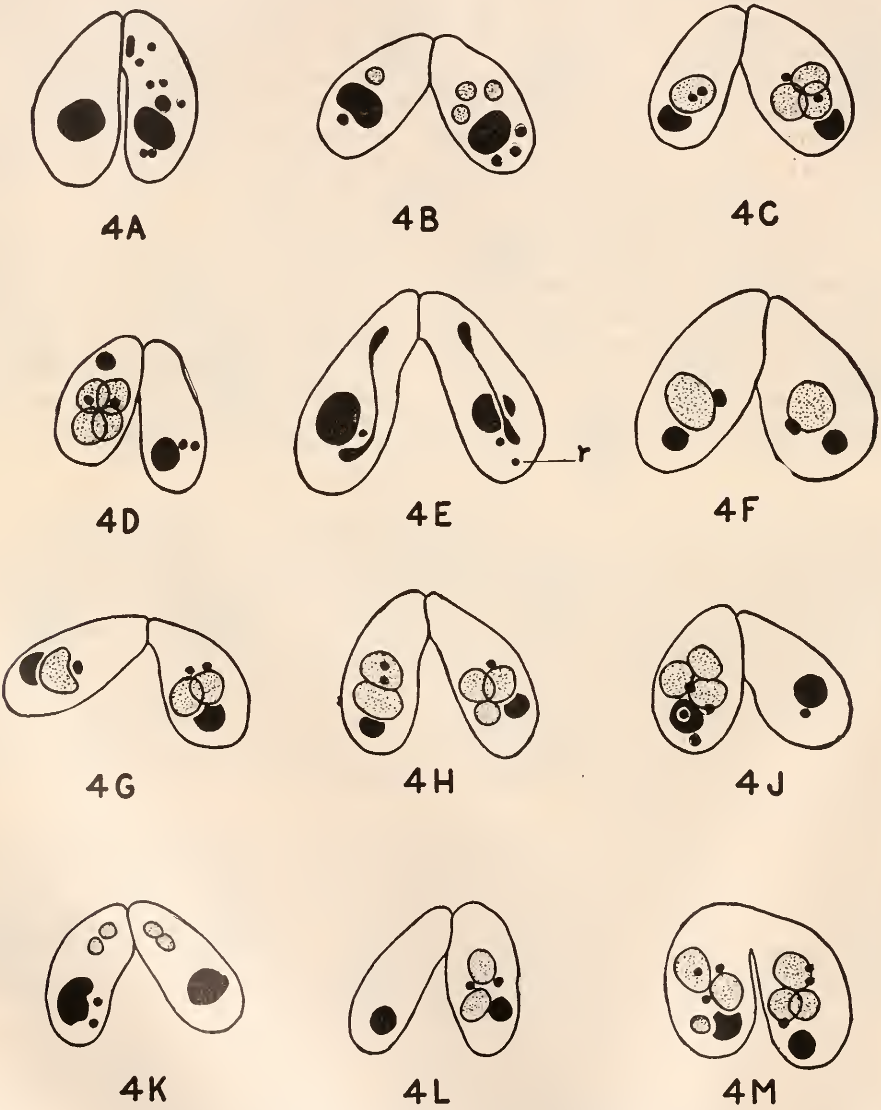

FIGURE 4. Abnormalities in conjugation induced by centrifugation. See explanation in text.

relic nuclei, a migratory nucleus and a stationary nucleus. This could be interpreted as conjugation between an amicronucleate and a bi-micronucleate cell were it not for the fact that *no* amicronucleate and *no* bi-micronucleate cells were observed elsewhere in the culture. Figure 4B is most readily interpreted as due to a mate-to-mate transfer after fertilization and presumably just after the first post-zygotic division. If this interpretation is correct, each nucleus continued to develop in its normal manner to give rise to a macronucleus and a micronucleus. Either of these cases could also be interpreted on the basis of a dual effect of the centrifugation—an elimination of nuclei from one cell and an induced extra division of nuclei in the other cell, but the usual compensation for the loss of nuclei in one cell by the addition of nuclei to its mate makes the mate-to-mate transfer interpretation more likely. In other cases where there is no compensation, the interpretation of nuclear loss or induced extra divisions may be more seriously entertained.

Mate-to-mate transfers at later stages, *i.e.*, after the differentiation of micronuclei and macronuclei, are more common. Figure 4C shows a pair in which one of the macronuclei from one cell was transferred to the other cell and Figure 4D shows a pair in which both new macronuclei were transferred from one cell into its mate. These observations show no evidence for cytoplasmic control of nuclear processes, but demonstrate clearly that relocation of nuclei in the cytoplasm does occur during centrifugation. Other, but rarer, types of abnormalities are more illuminating. Some of these are associated with mate-to-mate transfer and others are presumably due merely to alterations in the positions of the nuclei within a single cell.

2. *Alterations in the number of nuclei*

The most common abnormality observed—next to simple mate-to-mate transfer—was essentially like the spontaneous abnormality discussed above. In some cells only two new nuclei were formed in contrast to the usual four, and the new nuclei were larger than normal nuclei in comparable stages. Figure 4E shows a pair in which both members show a spindle like the typical spindle for the second post-zygotic division, but each pair shows only one such spindle. Figure 4F shows a similar pair at a later stage and Figure 4G shows a pair in which the anomaly occurred in only one cell of a pair. These abnormalities can be explained on two simple assumptions: (1) the cytoplasm at the posterior end of the cell determines that a nucleus under its influence will undergo a "final" division with a longitudinally oriented spindle, and (2) the centrifugation resulted in a fertilization nucleus being placed under this influence before it had the opportunity to undergo a normal first post-zygotic division. If this interpretation is correct, the spontaneous abnormality may be similarly explained on the basis of some unspecified environmental influence that causes the fertilization nucleus to arrive at the posterior end prematurely. An alternative interpretation would hold that the figures shown represent conjugants from which some of the nuclei have been removed by centrifugation. If this is true, the experimentally induced aberrations are not necessarily related to the spontaneous aberrations where nuclear loss seems unlikely.

3. *Alterations in nuclear development*

Another kind of aberration noted involves the change of a presumptive micronucleus into a macronucleus or of a presumptive macronucleus into a micronucleus.

Since it is not possible to follow any particular pair through conjugation, such alterations can be detected only as cells containing abnormal relative numbers of micronuclei and macronuclei. Figure 4H shows a pair which appears to satisfy expectations. One pair-member is normal, *i.e.,* it contains two micronuclei and two macronuclei. The other pair-member shows two well developed macronuclei and one typical micronucleus. The other new nucleus, presumably derived from the other presumptive micronucleus, is much larger, less deeply staining, and appears to be in the process of developing into a macronucleus. This may be interpreted as a nucleus whose developmental route was altered at a fairly late stage. Whether it would develop into a fully formed new macronucleus is of course not known. Figure 4J shows the reciprocal transformation, in this case combined with mate-to-mate transfer. One cell contains only a single micronucleus; the other contains four micronuclei and three new macronuclei. The pair as a whole has, therefore, produced five micronuclei and three macronuclei. It appears reasonable to assume that one of the presumptive macronuclei has given rise to a micronucleus. Since aberrations of this sort have been observed in the AA strains only when the cells are centrifuged, and since centrifugation certainly relocates the nuclei in the cytoplasm, it seems reasonable to assume that the relocation itself results in an alteration in nuclear development.

4. *Other alterations*

Other alterations in nuclear development have also been observed, but these are much rarer and an insufficient number of each kind has been observed to warrant extended discussion. A few of these will be illustrated. Figure 4K shows a pair in which the nuclei in one cell are normal, while the other cell contains only two nuclei—both developing as macronuclei. This can be interpreted by assuming two effects of the treatment—an alteration in nuclear number followed by the transformation of a presumptive micronucleus into a macronucleus. Since this pair had been centrifuged three times during conjugation, this interpretation does not seem improbable.

Figure 4L shows a pair consisting of one apparently normal cell and one with no new nuclei. This may be interpreted as due to the transfer of the fertilization nucleus from one cell into its mate followed by a single division of each of the nuclei. It could also be explained by a mate-to-mate transfer at an earlier stage be present, additional evidence for alteration in nuclear development is seen in the nuclei from one cell.

Figure 4M is more difficult to interpret since the total number of new nuclei in each cell is six rather than the usual four. This could be due to an extra division of one of the products of the first post-zygotic divisions in each cell. This result might also be found if this was originally a triple, the extra nuclei being derived from a third cell before it was lost. Regardless of how the multiple nuclei came to be present, additional evidence for alteration in nuclear development is seen in the small partially developed macronucleus in the left member.

DISCUSSION

Sonneborn (1951) pointed out several instances where cytoplasmic control of nuclear behavior was indicated in the cytogenetic processes of *Paramecium.*

Particularly he suggested that cytoplasmic locations were determinative in the following instances: 1) the survival or disintegration of haploid nuclei following meiosis; 2) the differences in the behavior of the migratory and stationary nuclei; 3) the differentiation of micronuclei and macronuclei. His conclusions may be extended with little modification to *Tetrahymena* and additional instances of cytoplasmic control may also be suggested.

In proceeding to a discussion of the factors involved in nuclear behavior at conjugation in *Tetrahymena*, it is necessary to point out the general features of this behavior. These may be discussed under the following headings: nuclear migration, nuclear division and nuclear differentiation.

1. *Nuclear migration*

The various stages of nuclear reorganization are characterized by events occurring in specificially localized regions of the cytoplasm. The first question raised is whether the movements resulting in the specific localizations are autonomous, or whether the cytoplasm controls these movements to some extent. This question may not be answered with certainty but certain considerations favor the latter solution. The location of the nuclei may be understood in terms of two migrations, the first a migration toward the anterior end of the cell (toward the contact membranes) and the second a migration toward the posterior end of the cell (away from the contact membranes). Before conjugation the micronuclei occupy positions near the macronucleus, but may be either anterior or posterior to it; the first and second pre-zygotic division figures are always anterior to the macronucleus. After meiosis the nuclei continue to move anteriorly until one of the haploid nuclei attaches to the membrane between the cells. This terminates the anterior migration; all subsequent movements, except those resulting from nuclear displacement during nuclear division, are from the anterior end of the cell to the posterior end. After one nucleus attaches, the relic nuclei begin to move posteriorly and, if they do not disintegrate first, come to lie at the extreme posterior end of the cell. The fertilization nucleus also migrates posteriorly from its position in front of the old macronucleus, and the new macronuclei move posteriorly from the position where they were placed by the elongated spindles of the second post-zygotic division.

The anterior migration is initiated at the time the cells come in contact at their oral surfaces; it is terminated at the time a nucleus attaches at these same surfaces. There is thus circumstantial evidence that events occurring at the contact surface determine nuclear migrations. Several possibilities are available in regard to the nature of such events, but in the absence of further evidence, speculation appears unprofitable.

2. *Nuclear divisions*

The nuclear divisions are as follows: two meiotic divisions, a pre-zygotic equational division of one of the haploid nuclei and usually two post-zygotic divisions. The meiotic divisions, like the nuclear migrations, are initiated following the attachment of the cells. The pre-zygotic equational division is clearly related to the cytoplasmic disposition of the nuclei since it occurs only in the nuclei attached to the contact membranes. The specificity of this cytoplasmic location is shown by the fact that attachment is always on the right side of the cell. The nature of these contact membranes, the manner in which they adhere to one another, the way they initiate the process of conjugation and control nuclear movements (if indeed they do), the manner in which they assure the transfer of presumably identical nuclei in opposite directions are all problems of importance in understanding the complex phenomena of conjugation. These problems require much further study.

The post-zygotic nuclear divisions are also controlled to some extent by the cytoplasmic conditions, but these conditions are not obviously related to the contact membranes. The first post-zygotic division occurs anterior to the old macronucleus; the second occurs posterior to the old macronucleus. Evidence has been presented which suggests that if the first post-zygotic division occurs posterior to the macronucleus, this is the final division, giving rise to a macronucleus and a micronucleus. Since the division occurring anterior to the macronucleus under normal circumstances shows a spindle oriented transversely in the cell, whereas all divisions occurring posterior to the macronucleus show spindles oriented longitudinally in the cell, the influence of this cytoplasm may lie in or be associated with its control of spindle orientation.

3. *Nuclear differentiation*

Several types of nuclear differentiation are noted during the conjugation process. The first differentiation is that of enlargement of the micronuclei prior to meiosis. Like the nuclear migrations and meiosis, this enlargement is directly or indirectly related to events occurring at the contact surfaces. The second differentiation is that occurring in the haploid nucleus attached to the membranes. The unattached nuclei disintegrate without dividing; the attached nucleus divides and both its daughter nuclei persist. Particularly it is to be noted that the daughter nucleus free in the same cytoplasm with the disintegrating nuclei does not distintegrate. It must, therefore, be different from them, though it is extremely unlikely that this difference is genetic; both types of nuclei are presumably haploid and must reasonably be expected to be alike in genetic material in many instances. The size difference between the stationary nucleus and the relic nuclei is further evidence for some kind of differentiation. Sonneborn (personal communication) suggests, on the basis of observations on *Paramecium,* that a transient cytoplasmic condition initiates the degeneration of free nuclei at a particular time, even though complete disintegration is not observed until later. According to this view, the stationary nucleus is released into the cytoplasm when the cytoplasm is no longer capable of initiating degeneration.

The differences in the behavior of the migratory and the stationary nuclei may also be ascribed to cytoplasmic relations, but it is possible that the differences are due simply to the fact that one is physically bound to the contact membranes while the other is free in the cytoplasm. One final difference between the stationary and the relic nuclei is seen in the fact that fertilization takes place between the incoming migratory nucleus and the stationary nucleus even if the relic nuclei are in the same cytoplasm. Perhaps it is premature, in the absence of genetic evidence, to conclude that the migratory nucleus *never* fuses with a relic nucleus, but this conclusion is certainly strongly indicated for other organisms that have been studied genetically (Sonneborn, 1947). It appears probable that the stationary nucleus is attracted to or attracts the migratory nucleus and that the nuclei are so differentiated that under normal conditions the relic nuclei cannot participate in the union.

There is apparently no major difference between the fertilization nucleus and one of its daughter nuclei. Either may divide once to give rise to a macronucleus and a micronucleus. The difference in their normal behavior is apparently due to their cytoplasmic location rather than to intrinsic factors. The differences in the sizes of the macronuclei and micronuclei produced directly by these two kinds of nuclei are not understood, but may be explained on the basis of a limitation of substrate for nuclear development in the conjugating cells. According to this view, a single macronucleus and a single micronucleus in the cytoplasm have more reserves to draw on and hence develop further than would two macronuclei and two micronuclei in the same cytoplasm. Other interpretations are possible, however.

The disintegration of the macronucleus at a particular time in the conjugation cycle may also be considered a type of nuclear differentiation, but no information is available concerning the factors involved.

The final nuclear differentiation is that which distinguishes the macronuclei from the micronuclei. The evidence presented demonstrates that this differentiation of nuclei is directly related to their positions in the cytoplasm at a critical time. The conditions at the anterior end of the cell are such as to bring about the development of macronuclei; the conditions at the posterior end cause the development of micronuclei. That the nuclei developing as macronuclei are not different in their potentialities from those developing as micronuclei is shown by the fact that presumptive macronuclei may be induced to become micronuclei and presumptive micronuclei may be induced to become macronuclei by altering the positions of the nuclei in the cytoplasm. This conclusion is further supported by evidence that under some circumstances the daughter nuclei produced at the first post-zygotic division can directly differentiate as macronuclei or micronuclei; under normal circumstances each of these nuclei gives rise to one macronucleus and one micronucleus. It would be difficult to explain these results on the basis of the segregation of genetic elements.

4. *Cytoplasmic differentiation*

Evidence for nuclear differentiation of various kinds is available in the account given above. Evidence for progressive cytoplasmic differentiation through conjugation is less readily obtained, but certain observations suggest that this also plays an important role. It is known, for example, that a diploid nucleus dividing immediately anterior to the old macronucleus undergoes meiosis during the initial stages of conjugation, but undergoes mitosis after fertilization in the same position. This could be explained by some kind of nuclear differences characterizing the nuclei at the different times, but it is equally possible that the cytoplasm has been altered. Similar considerations hold in regard to the behavior of nuclei at the anterior end of the cell at different times during conjugation. The nuclei produced after the second pre-zygotic division show no evidence for developing as macronuclei, while the nuclei produced after the second post-zygotic division and placed at the anterior end do develop as macronuclei. Other examples could also be drawn in which nuclear and cytoplasmic differentiation are equally probable as an explanation for the differences in the behavior of nuclei in the same cytoplasmic regions at different times. It appears probable that a progressive cytoplasmic alteration is correlated with a progressive nuclear alteration throughout conjugation and that an understanding of the process must include consideration of a complex interaction of nuclear and cytoplasmic factors.

Although the observations on *Tetrahymena* demonstrate an important influence of the cytoplasm on nuclear behavior, it cannot be concluded that the cytoplasmic conditions are not ultimately under the control of the nuclei. Observations on *Paramecium* (Sonneborn, 1951; Nanney, 1953) indicate that certain cytoplasmic conditions controlling nuclear development are determined by the nuclei and that cellular differentiation may proceed as a series of inter-determinations of the nucleus by the cytoplasm and of the cytoplasm by the nucleus. It is probable that many aspects of nuclear behavior, though immediately under the control of the cytoplasm, are ultimately traced to nuclear activity.

SUMMARY

1. The details of the nuclear processes occurring at conjugation in certain selfing strains (AA strains) of *Tetrahymena* are presented with an experimental analysis of certain of the factors influencing nuclear behavior and nuclear differentiation.

2. While it is not possible at the present time to describe in detail the mechanisms operating to assure an orderly sequence of events, it is clear that the cytoplasm plays a critical role in directing the activities of the nuclei. This is demonstrated by two facts: that the various events are specifically localized in the cytoplasm and that experimental alterations in the positions of the nuclei result in alterations in nuclear behavior.

3. It is concluded that the entire conjugation cycle proceeds as a complex series of nucleo-cytoplasmic interactions.

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FRESH-WATER POLYCHAETOUS ANNELID, MANAYUNKIA SPECIOSA LEIDY, FROM LAKE ERIE

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A collection of some 23 specimens of a very small fresh-water sabellid, collected in Lake Erie by George M. Moore, was presented to me for examination. They proved to be specimens of the interesting fresh-water sabellid polychaete genus, *Manayunkia* Leidy, which is very closely related to *Fabricia* Blainville consisting of marine species. There has been some confusion as to whether one or two species are involved—*M. speciosa* Leidy, 1858, and *M. eriensis* Krecker, 1939.

Manayunkia speciosa was originally described from specimens obtained from the Schuylkill River at Fairmount, Philadelphia (Leidy, 1858, 1883)—the first record of a strictly fresh-water polychaete. They were found in tubes of mud attached to stones. Additional specimens were found in Egg Harbor River, New Jersey, attached to a fragment of pine-bark. Further observations were made by Potts (1884) and Foulke (1884).

Meehean (1929) found specimens of *Manayunkia* dredged in the western end of Lake Superior (Duluth Harbor). Under the name of *M. speciosa,* he added to the original description, principally in regard to the collarette; he observed only three pairs of haemal loops, one pair in each of the last three segments, instead of a pair in each segment as indicated by Leidy. Krecker (1939) found specimens of *Manayunkia* in Lake Erie, 30 miles due east of Put-in-Bay, at a depth of 55 feet. He considered that the specimens from Lake Erie were the same species as those from Lake Superior but differed sufficiently to be considered distinct from *M. speciosa* and gave the name of *M. eriensis* for the Great Lakes specimens. Hartman (1951), in a review of the subfamily Fabriciinae, indicated that *M. eriensis* Krecker might be the same as *M. speciosa* Leidy. Zenkevitsch (1925), in a detailed study of *M. baicalensis* (Nusbaum) from Lake Baikal, pointed out the general characters of the group to which *Manayunkia* belongs, as well as some errors in the original description of *M. speciosa*.

Based on a study of the Lake Erie specimens, there seems to be only one species involved and *M. eriensis* should go into synonymy. There follows a summary of the description. The specimens are deposited in the United States National Museum (Cat. No. 24736).

FAMILY SABELLIDAE

SUBFAMILY FABRICIINAE

TRIBE THORACOGENEATA ZENKEVITSCH, 1925

GENUS MANAYUNKIA LEIDY, 1858

MANAYUNKIA SPECIOSA LEIDY, 1858

Figure 1, A–K

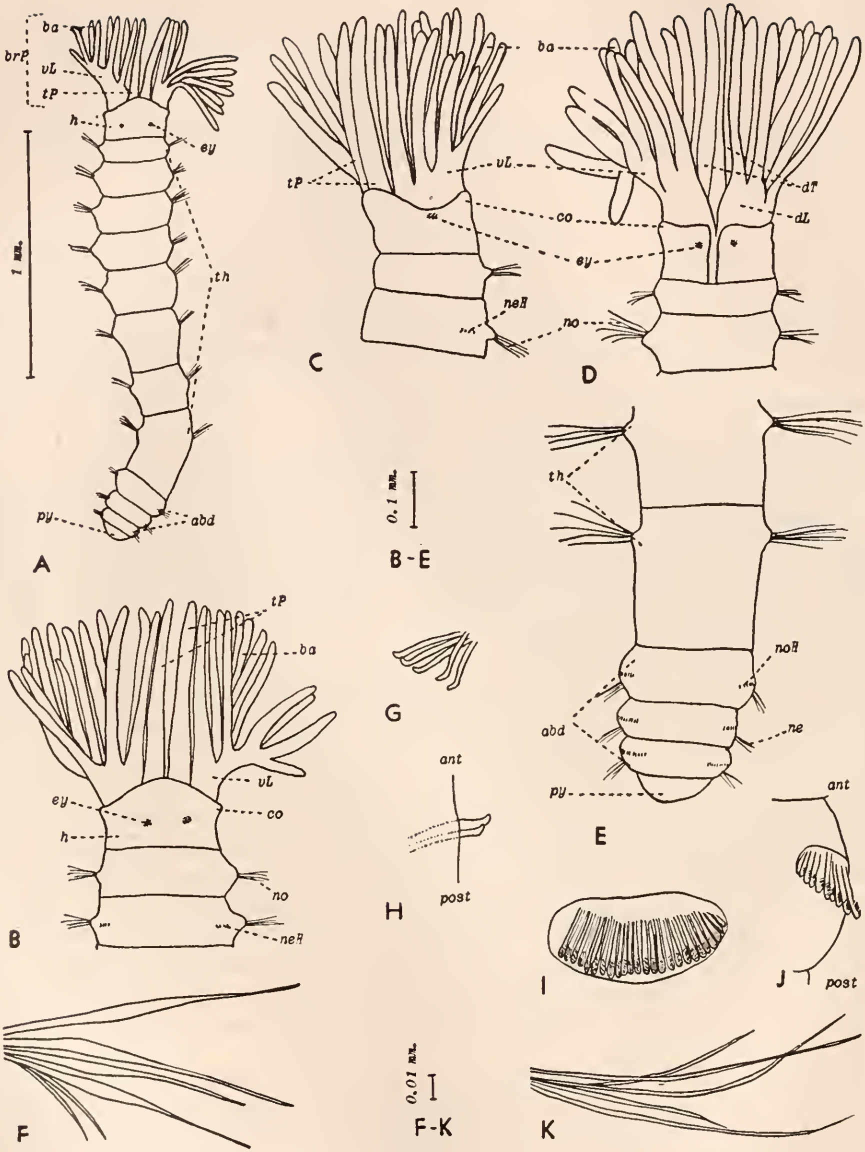
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FIGURE 1. *Manayunkia speciosa* from Lake Erie: A, ventral view of entire worm; B, ventral view anterior end; C, lateral view anterior end; D, dorsal view anterior end; E, dorsal view posterior end; F, thoracic notosetae; G, thoracic neuropodial hooks; H, same, showing position in body; I, abdominal notopodial hooks; J, same, showing position in body; K, abdominal neurosetae. abd, abdominal setigerous segments; ant, anterior; ba, ciliated barbules or tentacles; brP, branchial plume or prostomial tentacular crown; co, collarette; dL, dorsolateral inner branch of lateral lobe; dT, mediodorsal tentacles; ey, eyespot; h, "head"—fused prostomium and achaetous first segment; ne, neurosetae or ventral setae; neH, neuropodial hooks; no, notosetae or dorsal setae; noH, notopodial hooks; post, posterior; py, pygidium or anal segment; th, thoracic setigerous segments; tP, tentacular palps; vL, ventrolateral outer branch of lateral lobe.

Manayunkia speciosa Leidy, 1858: 90; 1883: 204–212, Pl. 9, Figs. 1–13.—Potts, 1884: 21–22.—Foulke, 1884: 48–49.—Zenkevitsch, 1925: 36–38.—Meehean, 1929: 479–480.—Hartman, 1951: 389.

Manayunkia eriensis Krecker, 1939: 153.—Hartman, 1951: 389.

Size. Length 2.5 to 4.9 mm., width up to 0.3 mm. In life, Lake Erie specimens 2.8 to 3.3 mm. long. In a preserved specimen 2.5 mm. long, length of branchial plume is 0.4 mm.; head and thoracic region, 1.85 mm.; abdominal region and pygidium, 0.25 mm.; greatest width, 0.3 mm.

Description. Body very small, almost cylindrical, slightly attenuated posteriorly, with segments few in number (12 segments—first achaetous, 8 thoracic setigerous, three abdominal setigerous; Fig. 1, A). Prostomial tentacular crown or branchial plume (Fig. 1, A–D) consisting of : (1) a pair of semicircular lateral lobes (lateral lophophores of Leidy), each pair branching near the base—a diagonal ventrolateral outer branch with four pairs of ciliated barbules or tentacles and a diagonal dorsolateral inner branch with 5 pairs of ciliated barbules; barbules on both branches longest ventrally (basal end of branch), gradually become shorter in more dorsal position; barbules reach about the same distal level—as if they had been cut off; (2) a pair of mediodorsal tentacles; (3) a pair of medioventral tentacles—the tentacular palps, which are slightly larger than and subequal in length to the other tentacular appendages; they receive branches of the blood vascular system (the only ones of the tentacular prostomial appendages that have blood vessels); in life, they are conspicuous by the bright green blood pulsating rhythmically, contracting and expanding longitudinally, thus alternatingly contracting and dilating the blood vessels. Thus, there are 20 ciliated tentacles on each side, 40 altogether in the branchial plume. [Leidy, in the original description, indicated that the lateral lophophores were simple, not bilobate; this was questioned by Zenkevitsch (1925, p. 37), since in *M. aestuarina* and *M. baicalensis* each lophophore is bilobed; close examination shows that this is also the case in *M. speciosa.*] There may be 6–10 brownish pigment spots on each side near the bases of the barbules (not visible when preserved; according to Meehean, younger ones have few or none; Leidy, Pl. 9, Fig. 1).

Prostomium fused with first achaetous segment (conveniently referred to as the "head"), with a pair of eyespots near the bases of the tentacular palps, with a distinct collarette—on the ventral side, projected anteriorly into a rounded lobe; open and separated middorsally; collarette may be somewhat flared (Fig. 1, B–D; Leidy made no mention of a collarette although the ventral extension of it was shown on his Fig. 1; it was noted by Meehean). Anterior thoracic region consists of 8 setigerous segments—first with dorsal setae only (notosetae), remainder with additional long-handled ventral uncini or podal hooks (neuropodial hooks). Thoracic notosetae 5–8 in number, limbate, with capillary tips (Fig. 1, F; Leidy, Pl. 9, Figs. 3–4). Thoracic neuropodial hooks 3–5 in number, with long curved handle, ending in small recurved hook directed anteriorly (Fig. 1, G–H; Leidy, Pl. 9, Fig. 5). Posterior abdominal region consists of last three setigerous segments (Fig. 1, E), with dorsal long-handled uncini or podal hooks (notopodial hooks) and ventral setae (neurosetae). Abdominal notopodial hooks consisting of numerous (14–30) small hooks arranged in a close transverse row; hooks with broad handle ending in expanded pectinate tip—numerous very fine teeth in several rows on one side, directed anteriorly (Fig. 1, I–J; Leidy, Pl. 9, Figs. 6–7). Abdominal neurosetae 3–5 in number, slender, with capillary tips (Fig. 1, K). Anal segment or pygidium short, rounded, without eyespots (Fig. 1, E).

Food brought to funnel-like, capacious mouth by ciliary currents of the branchial barbules. Digestive tract a median tube, alternately dilated within the segments, ciliated for the whole length (Leidy, Pl. 9, Fig. 1). Anus opens ventrally between last segment and pygidium. Excrement passed forward in the tube, aided by the longitudinal ciliated groove—midventral in abdominal region, passing diagonally on one side, middorsal in thoracic region. Blood vascular system, containing green blood, consists of a sinus around the digestive tract (corresponds to the dorsal blood vessel) in which the blood passes anteriorly by contractions of the gut wall, two branchial "hearts" at the bases of the branchial lobes, two vessels in the tentacular palps, a ring of vessels near the mouth, a vessel ventral to the digestive tract, paired loops in each segment from the ventral blood vessel to the intestinal sinus, the last three pairs especially conspicuous. A pair of looped nephridial tubes in segments 1–2 (these paired large elliptical organs were suspected by Leidy to be the testes, although he stated that he did not examine their structure).

Sexes separate (not hermaphroditic as indicated by Leidy; see Zenkevitsch, 1925). Females with ovaries and eggs in different stages of development in segment 5 (setigerous segment 4); eggs escape through opening between segments 5–6 (Meehean, 1929). Eggs laid within the tube where they pass their early developmental stages; young retained until they have at least 8–9 setigerous segments, with eyespots present, with 4–5 pairs of barbules on the branchial plume (Leidy, Pl. 9, Figs. 8–13). On the mature females, there may be a thickened semicircular band on the ventral side of the anterior part of segment 7 (setigerous segment 6; this expansion of the forepart of segment 7 suggested to Leidy that it might be the production of a head prior to the transverse fission of the worm, although he did not observe the process; Pl. 9, Fig. 1; Zenkevitsch showed that it was present only in mature females and formed part of a brood chamber between segments 7 and 9). Males with testes and sperm in segments 7–9 (setigerous segments 6–8; not in segments 1–2 as indicated by Leidy—these structures proved to be the paired nephridia as indicated above). (In *M. baikalensis,* a mediodorsal sperm duct passes from segments 6–9, conducting the sperm to the head region; opens by genital pore near the base of the head behind the nephridiopore; the anterior part of the sperm duct with distinct enlargement filled with sperm, forming a seminal vesicle; details not worked out for *M. speciosa.*)

Color (preserved): without color or may be darkly pigmented in part of head segment; (living): translucent olive-green, slightly brownish around the branchial lobes. Tube of mud—fine particles agglutinated by mucoid secretion, feebly annulated, cylindrical, straight or bent, sometimes branched (may be 2–5 branches), attached to fixed objects, the greater part free, pendant; tube may reach a length of several times that of the worm. (The specimens from Lake Erie were out of their tubes but some small empty tubes were found in the same haul.)

Locality. Lake Erie, 22 miles east of Put-in-Bay, Ohio, depth of 55 feet, July 13, 1951, collected by George M. Moore. Dredged by fine screened trawl on bottom of gray clayish mud with numerous dead shells. Some 23 specimens found in washings from the dredge, along with hydras, planarians, oligochaetes, and snails.

Distribution. North American fresh waters: rivers of southeastern Pennsylvania (Schuylkill River, Philadelphia) and southeastern New Jersey (Egg Harbor River); Great Lakes—Lake Erie (near Put-in-Bay, Ohio, 55 feet) and Lake Superior (Duluth Harbor, Minnesota, dredged). Probably much more widely distributed but, due to its small size, escapes notice.

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SOME ASPECTS OF PHYSIOLOGICAL AGING IN THE ADULT WORKER HONEY BEE[1, 2]

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In reviews on the general physiology of aging, Lansing (1947, 1951) has emphasized the need for study at a cellular level of the problem of senescence, a phenomenon demonstrating a uniform pattern in a wide variety of organisms. This concept encompasses examination of anatomical and morphological, degenerative alterations, as well as biochemical changes such as hormonal and enzymic perturbations accompanying the involutionary process of growing old. Such studies must necessarily involve a long-term investigation of the various facets of the phenomenon of aging in animals or individuals whose complete medico-ecological history is a matter of record. To this end the worker honey bee (*Apis mellifera*) represents an animal which can be obtained in large numbers and which can be maintained during its life span under controlled physical environmental conditions with a minimum of care, while occupying relatively little space.

In an earlier study, Rockstein (1950) reported that the number of cells at two representative levels of the brain of the adult worker bee decreased steadily from the day of emergence to old age, whereas the activity in total brain homogenates of the enzyme cholinesterase rose during the first week to ten days following emergence and remained at this elevated level throughout the remainder of the life of the bee. This indicated the absence of a significant role by this enzyme system in the physiology of aging, from the standpoint of senescence.

Important in many aspects of intermediary metabolism, specifically in vital processes like nucleic acid and carbohydrate metabolism (see Moog, 1946 and Roche, 1950), the phosphomonoesterases suggested another enzyme system of sufficiently elevated importance which might prove related to the process of senescence. In pursuing this problem further, the writer has therefore studied changes in activity of acid and alkaline sodium β-glycerophosphatases in total body homogenates, as a function of age in the adult worker honey bee, *Apis mellifera* L., the results of which studies are presented herewith.

METHODS

Frames of sealed worker brood were removed from a colony in the college apiary to an incubator maintained at 32.5° C. Adult bees in large numbers (2000 or more) were removed within 24 hours after emergence and were maintained

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thereafter in a large screen cage, under conditions of continuous artificial lightining and at a constant temperature of 26.5° C. and relative humidity of 50%, with adequate pollen, honey and water supplies for *ad libitum* feeding. At definite intervals of time, bees of known age were removed and homogenized in lots of twenty by the procedure described by Rockstein and Herron (1951), and the acid and alkaline phosphatase activity determined, respectively, as described by Rockstein and Levine (1951) and Rockstein and Inashima (1953). Determinations on very old bees were deferred until the point where the cage population began to show a rapid decline and where just enough live material was available.

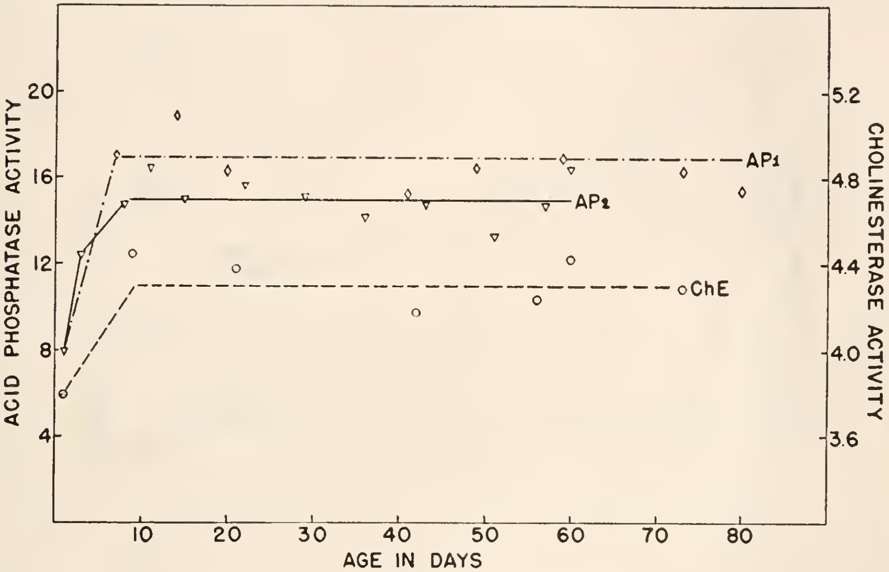

FIGURE 1. Acid phosphatase in whole body and cholinesterase in whole brain homogenates of the adult worker honey bee.

RESULTS

Figure 1 shows two curves labelled "AP_1" and "AP_2", denoting acid phosphatase studies of whole body homogenates for two populations of bees, as compared to data from the earlier report by the author (1950), averaged and plotted for cholinesterase activity in whole brain homogenates. Phosphatase activity is expressed as micrograms of phosphate as phosphorus, released in 1½ hours at 35° C. at pH 5.4, in a 0.2 ml. sample of deproteinized incubation mixture (see Rockstein and Levine, 1951, for details); cholinesterase activity is expressed as micromoles of acetylcholine bromide hydrolyzed per whole brain per hour at 30° C. During the first eight to ten days of adult life the total body acid enzyme shows a rise in activity representing an increase of about 90%; during the corresponding period the total brain cholinesterase shows a parallel rise in activity, but of only 14%. For both enzymes this elevated activity appears to remain undiminished from about ten days to very old age.

Figure 2 shows similar curves for the alkaline enzyme (only one series) and collated data for brain cell number, from the author's earlier report (1950), averaged and plotted for comparison. Alkaline enzyme units are the same as for the acid enzyme, representing activity under identical conditions except that the pH of incubation was maintained at 8.1. Here is seen a steady decrease in brain cell number to the extent of about a 35% decline in old bees from the original cell count in day-old bees; total body alkaline enzyme, however, shows a steady decline during the first week to ten days to a level 44% below that of day-old bees, following which no significant change occurs until old age.

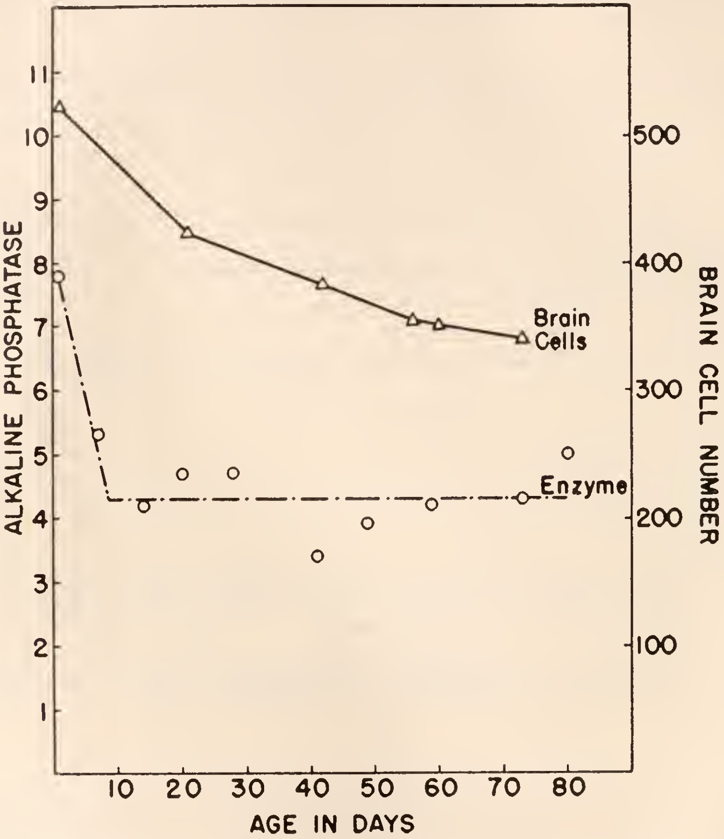

FIGURE 2. Alkaline phosphatase in whole body homogenates and brain cell number in the adult worker honey bee.

Each point plotted for phosphatase activity in both figures represents the median value for at least five replicate determinations from each homogenate preparation.

DISCUSSION

If the total aging process is accepted to be an outward manifestation of inner biochemical alterations at the cellular level, the immediately controlling process or processes may be more precisely defined in terms of modifications in the activity of identifiable enzyme systems catalyzing important metabolic processes. One agrees that correlation of data, whether it be statistically or graphically demonstrable, does not guarantee causality for phenomena under consideration, biological or otherwise. However, as in the case of cholinesterase, an enzyme important in the metabolism of acetylcholine (itself important in the mediation of the nerve impulse in certain parts of the nervous system of most animals), deductions can be made with some degree of certainty concerning their rationality.

From the earlier study by Rockstein (1950), no such positive relationship was indicated between senescence and brain cholinesterase in the honey bee, but rather some positive part played by that enzyme in post-embryonic maturation of the neuromotor mechanism.

Data in this report indicate no direct relationship between the alkaline and acid phosphomonoesterases and senescence; however, if the alkaline is indeed involved its effects must be quite indirect; *i.e.,* its decline at an early stage of adult life may be the forerunner of a gradual biochemical decline eliciting more directly the outward manifestations of senescence. This suggestion is in harmony with the concept of aging as a process initiated rather early in post-embryonic life of an individual and proceeding through several phases which include progressive maturation and senescence.

As has been mentioned elsewhere (Rockstein and Herron, 1951), Moog (1946) proposed that the presence of acid and alkaline phosphomonoesterases signifies a dual, matching dephosphorylating mechanism in the intermediary metabolism of glycogen. In the adult worker honey bee, data presented in this report point to a singular reciprocal relationship between the acid and alkaline sodium β-glycerophosphatases; *i.e.,* the acid enzyme shows a rise in activity during the first week to ten days following emergence while the alkaline enzyme shows a corresponding fall in activity during this same period of time. The fact is that this represents a post-larval period in this species, during which the power of flight is apparently being developed to its maximum. Wing beat frequency in *Drosophila* is reported by Sacktor (1950) as having been found by Chadwick and Williams (unpublished data) to be low immediately following emergence and to increase during the first few days of adult age to a level maintained throughout adult life; Sacktor himself found a marked increase in the activity of cytochrome oxidase, in total body homogenates of DDT-resistant and normal strains of the common house fly, the second day after adult emergence. The fact that brain cholinesterase in the adult honey bee also shows a strikingly parallel increase during the first week to ten days of adult life suggests that the interrelationship among the three enzyme systems, mentioned for the honey bee in this report, may be part of a well-integrated pattern of biochemical alterations concerned with completion of development of adult characteristics, particularly the neuromotor mechanism of strong-flying species. In this connection, evidence has been presented in an earlier discussion by Rockstein (1950), as well as in reports by Nachmansohn (1939), Sawyer (1943a, 1943b, 1944), Welsh and Hyde (1944) and Lindeman (1945), concerning the correlation between cholinesterase activity and the attainment of the ability to perform rapid movements in whole embryos, in developing immature young, and in different species of animals with varying degrees of motor ability.

Watanabe and Williams (1951) reported that the cytochrome oxidase activity in isolated sarcosomes (giant mitochondria) of the flight muscles of *Phormia regina* showed a pronounced drop (about 50%) during the first three to four days following adult emergence, at which reduced level of activity the enzyme in question remained constant thereafter. The variance between their data and those of Sacktor (1950) for total homogenates of the house fly indicates a need for further study of this enzyme in the house fly at the tissue level. Watanabe and Williams also reported a precipitous decline in catalase activity of sarcosomes during the similar three-four day period following emergence to about 20% of the original activity of a newly emerged adult fly, which decline was followed by a gradual dropping off to a low level of about 10% of the original activity at the end of the third week of adult life. The pattern of the latter findings suggests a possible basis for re-examination of the biochemical picture in the honey bee with regard to catalase, as well as the phosphatases, at the organ or tissue level, for possible further clarification of the maturation process during the post-emergence period in this species, and in other insects with a strong flight pattern.

SUMMARY

1. Acid sodium β-glycerophosphatase in whole body homogenates of adult worker honey bees shows a rise in activity by about 90% from the first to the tenth day following emergence and remains unchanged thereafter at this elevated level until old age.

2. Alkaline phosphatase shows a steady decline in activity to about 44% below that of day-old bees, by about the tenth day following emergence, remaining unchanged at the lowered level until old age.

3. Although no direct relationship between these enzymes and the process of senescence is apparent, the correspondence between these data and earlier findings is discussed in terms of the post-emergence development of the neuromotor mechanisms in vigorous-flying holometabolous insects.

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EMBRYONIC DEVELOPMENT OF THE LORDOTIC AND NORMAL GUPPY, LEBISTES RETICULATUS (PETERS)[1]

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Previous studies have shown that the mutation resulting in lordosis of the vertebral column in *Aplocheilus latipes* (Aida, 1930) and in *Lebistes reticulatus* (Kirpichnikov, 1935; Harrison, 1941; Goodrich *et al.*, 1943; Rosenthal and Rosenthal, 1950) behaves as a recessive, autosomal single factor Mendelian character. The lordotic condition becomes progressively more pronounced as the fish ages and may cause swimming activity to be somewhat laborious and erratic. Growth and development of either sex and reproduction in the female do not appear to be affected. However, the fertilizing capacity of sperm from mature mutant males is reduced (Rosenthal, 1951).

Since the spinal curvature is markedly apparent in the new-born young of *Lebistes,* it was of interest to compare the growth and development of the lordotic and normal embryo as an introduction to the study of the biochemistry of the lordotic mutation.

MATERIALS AND METHODS

Normal fish, obtained from a local tropical fish hatchery,[2] were maintained in the laboratory for variable lengths of time before use. The lordotic fish were raised and maintained under conditions similar to those previously reported (Rosenthal and Rosenthal, 1950; Rosenthal, 1952). As female fish approached parturition, they were isolated to individual jars containing masses of *Ceraptopterus* until the brood was born. The new-born young were removed within 3 to 20 hours, blotted dry on filter paper and the entire brood weighed to the nearest 0.1 mg. The young were then dried to constant weight at 105° C. to determine their water content. The dry residue was next extracted with ten 3-ml. portions of ethyl ether and the fat-free residue was dried an additional 24 hours. The fat content of the material was calculated by difference. A series of embryos[3] was obtained by sacrificing female fish at weekly intervals following the birth of a brood. The fish were killed by dropping them into boiling water since heat coagulation was found to be the best method for removing the ova or embryos from the ovary without breaking them. The ovarian contents were treated in the same manner as new-born young. Embryos or young that were not normally developed were discarded.

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[2] Normal fish and live food such as Tubifex worms were generously supplied by Messrs. William Schaumberg and John Detrich, of Crescent Fish Farms, New Orleans, to whom the author is indebted.

[3] Since the embryo cannot be readily separated from the yolk of the egg, the entire embryo-yolk complex was removed and treated as a unit and this combination of embryo and yolk will be considered interchangeably, for the purposes of this report, as either the ovum, embryo-yolk complex or embryo depending on the data to be discussed.

For chemical analysis, quantities of dried, fat-free material weighing 15 to 40 mgs. from individual broods of young or groups of ova and embryos were digested by boiling with one ml. concentrated sulfuric acid containing 4 drops of 5% copper sulfate. The digests were completely cleared by the addition of a few drops of 30% hydrogen peroxide. After cooling, the digests were diluted to 10 ml. and suitable aliquots were taken for the determination of calcium by the method of Kramer and Tisdall (1921). Phosphorus was determined on aliquots of the digest by the method of Fiske and Subbarow (1925) and nitrogen by direct nesslerization. Nitrogen values were converted to protein by use of the factor 6.25. Duplicate determinations of calcium agreed within 6% and those for phosphorus and nitrogen within 1%.

RESULTS

A comparison of the data, shown in Table I, on the growth rate, fat and water content of the normal and lordotic *Lebistes* indicates no essential differences with the exception that the lordotic embryo is consistently the smaller. In view of the similarity between the two strains, the data will be discussed together.

Although the variation between groups of embryos is quite large as shown by the standard deviations, it can be seen that the wet weight of maturing ova remains essentially constant for the first seven days but increases rapidly from the time of fertilization until the embryo is born. The increase in weight is due to an in-

TABLE I

Embryonic growth of Lebistes reticulatus

Age, days	No. of embryos	No. of deter.	Average weight (mgs.)			H_2O % wet wt.	Fat % dry wt.
			Wet	Dry	Fat free		
			Normal				
0	185	5	2.96±0.73*	1.46±0.36*	1.14±0.20*	50.6	21.6
7	154	6	2.75±0.40	1.31±0.28	1.01±0.19	53.1	22.5
14	187	6	3.86±0.57	1.33±0.33	1.01±0.31	70.9	24.3
21	178	7	4.95±0.44	1.24±0.25	0.95±0.11	75.0	23.4
Term	279	23	5.64±0.69	1.22±0.20	0.96±0.15	78.4	19.5
			Lordotic				
0	47	4	2.06±0.61	1.00±0.28	0.73±0.23	51.6	26.7
7	85	3	2.56±0.12	1.23±0.05	0.94±0.03	52.2	23.5
14	92	8	3.14±0.93	1.29±0.21	1.02±0.19	63.1	21.8
21	50	3	4.06±0.38	1.07±0.18	0.83±0.15	73.8	23.2
Term	108	25	4.53±0.56	0.98±0.20	0.84±0.13	79.2	14.1

* $\sigma = \sqrt{\dfrac{\Sigma d^2}{N - 1}}$.

creasing water content which rises from an initial value of 50% to a final value of 80%. In contrast, the dry and the fat-free dry weight remain essentially constant throughout the development of the embryo. The fat content of the embryo, however, decreases slightly after the young are born. This probably reflects a utilization of stored yolk material during the period immediately following birth, since newly born poeciliid fish have rarely been observed to accept food during the first few hours after birth.

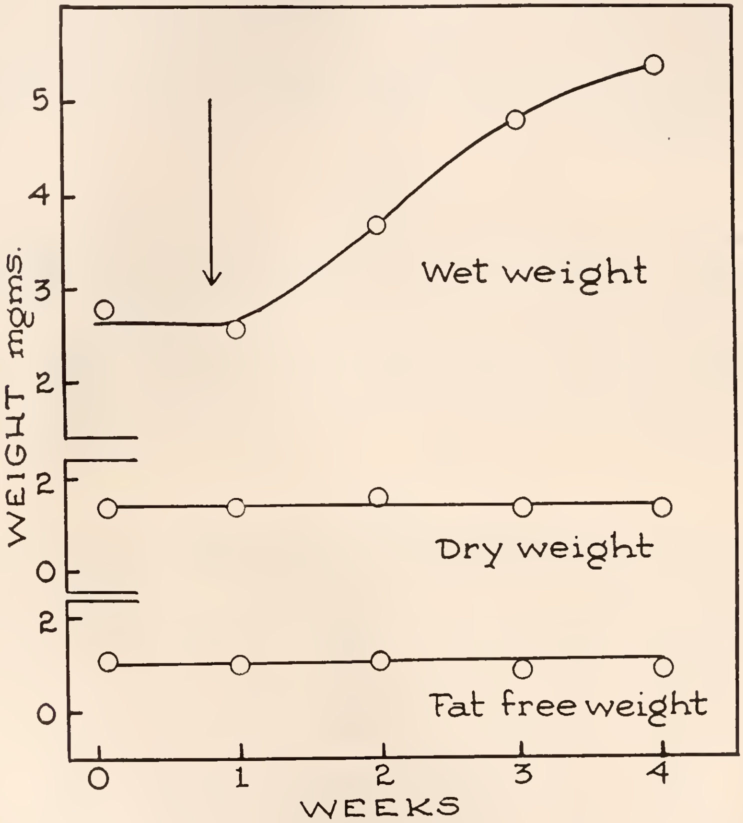

FIGURE 1. Average growth curves for lordotic and normal embryos of *Lebistes* during the brood interval. The arrow indicates the approximate time of fertilization of the ovum. See Table I.

In view of the similarity of the lordotic and normal embryo, the wet, dry and fat-free dry weights have been averaged and plotted against time of development as shown in Figure 1, to demonstrate graphically the growth rate of the *Lebistes* embryo.

calcium and phosphorus content of the dry fat-free tissue during growth and development of the *Lebistes* embryo. It is apparent that the total protein remains

The data presented in Table II show the changes that occur in the nitrogen, essentially constant for both the lordotic and the normal strains although the values obtained for the lordotic strain are somewhat smaller due to the smaller size of the embryo. The per cent of protein is also the same for both strains and remains essentially constant during gestation. The average total protein of the embryo-yolk complex accounts for 71.1 per cent of the fat-free dry weight (range = 67.1 – 76.1 per cent). The remainder consists of minerals and non-proteinaceous organic matter.

The calcium and phosphorus concentrations of the embryos present a somewhat different picture. During the first three weeks of development, the calcium concentration remains essentially constant but increases rapidly during the last week of gestation. The phosphorus concentration, on the other hand, increases slightly throughout the brood interval. It can be seen that the Ca/P increases only slightly during the early phases of gestation but increases rapidly during the last week preceding birth. The increasing calcium content is presumably associated with the incorporation of mineral elements in the osseous tissues of the body.

TABLE II

Embryonic growth of Lebistes reticulatus

Age, days	No. of deter.	Protein		Calcium	Phosphorus	Ca/P
		Total mgs.	% Fat-free dry weight			
			Normal			
0	4	0.77	67.7±3.7*	0.62±0.21*	1.18±0.07*	0.43
7	5	0.71	70.9±5.0	0.81±0.18	1.50±0.08	0.54
14	6	0.70	69.7±3.2	0.76±0.06	1.52±0.07	0.50
21	6	0.70	73.3±2.1	1.08±0.29	1.60±0.60	0.67
Term	12	0.70	73.3±3.9	2.24±0.40	1.90±0.12	1.17
			Lordotic			
0	2	0.50	68.6±4.3	0.95±0.05	1.35±0.05	0.70
7	3	0.65	68.8±0.8	0.73±0.04	1.46±0.05	0.50
14	4	0.74	72.8±1.8	0.97±0.12	1.49±0.08	0.65
21	3	0.63	75.8±4.3	0.93±0.12	1.66±0.09	0.58
Term	8	0.64	76.1±7.9	2.58±0.51	1.92±0.24	1.37

$* \sigma = \sqrt{\frac{\Sigma d^2}{N - 1}}.$

Since no essential differences between the lordotic and normal strains are evident, the data in Table II have been averaged for graphic presentation (Fig. 2).

DISCUSSION

The interval between the birth of successive broods of young for most poeciliid fish in general and *Lebistes* in particular approximates a period of 28 to 30 days. A brood interval may be divided into two separate phases. The first phase consists of 5 to 7 days in which the ova mature to a fertilizable stage (Hopper, 1943;

Rosenthal, 1952; Turner, 1937). The remaining 23 to 25 days, the second phase, may be considered as the period of gestation. The cyclical brood production makes it possible to obtain a graded series of embryos in similar stages of development.

It is apparent from these data that no essential differences exist in the rate of growth of the lordotic and normal *Lebistes* embryo as determined by the methods used. However, Harrison (1941) analyzed the total body calcium of adult mutant and normal *Lebistes* and found that the mutant strain had a higher calcium content. Additional data (in manuscript) obtained in this laboratory indicate that the vertebral column of the lordotic strain contains about 20% more calcium than the normal.

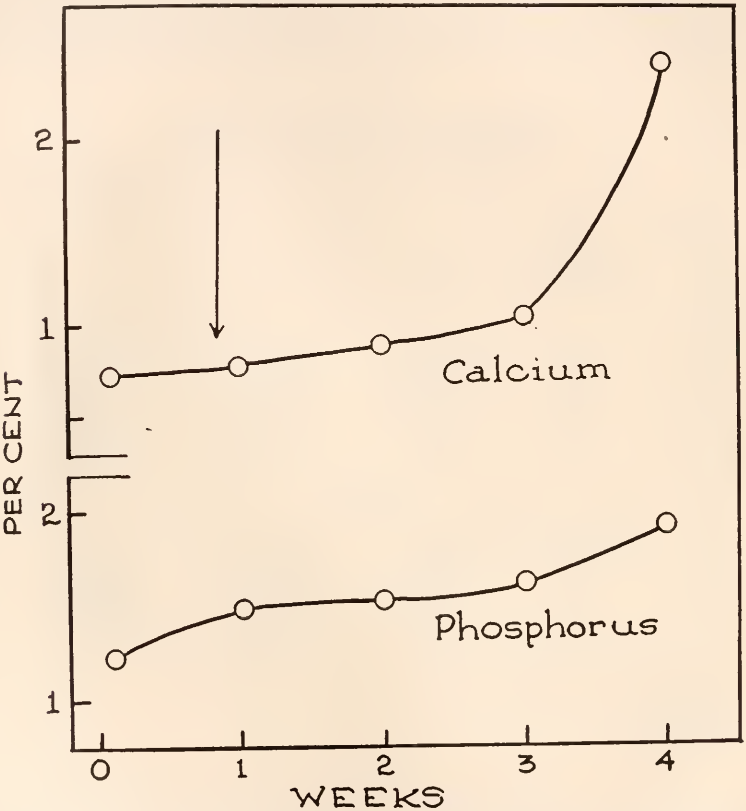

FIGURE 2. Average incorporation of calcium and phosphorus for lordotic and normal *Lebistes* during the brood interval. The arrow indicates the approximate time of fertilization of the ovum. See Table II.

Scrimshaw (1944; 1945) has studied extensively the embryonic growth pattern of various members of the family Poeciliidae and has concluded, on the basis of the constant dry weight of the embryo yolk complex, that an exchange of nutritive material must occur between the embryo and the tissues of the parent to replace materials lost through metabolic and excretory processes. The increasing calcium and phosphorus concentrations of the growing embryo, as shown in the present report, indicate that certain materials are obtained from the parent. It is conceivable that organic compounds can also diffuse across the ovarian membranes and, likewise, metabolic products formed during embryogenesis may diffuse through these membranes to be excreted by the parent.

The author wishes to thank Dr. William B. Wendel for his encouragement during the study and his aid in the preparation of this report.

SUMMARY

1. No differences could be established in the embryonic development of the lordotic and normal *Lebistes*.

2. The water content of developing embryos increases during gestation, but the dry and fat-free dry weights remain constant as does the protein concentration.

3. The calcium concentration, associated with calcification of osseous tissue, increases markedly during the last week of gestation while the phosphorus concentration increases only slightly during the entire brood interval.

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DEPHOSPHORYLATION OF ADENOSINE TRIPHOSPHATE BY TISSUES OF THE AMERICAN COCKROACH, PERIPLANETA AMERICANA (L.)

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The recent demonstration (Albaum and Kletzkin, 1948; Calaby, 1951) of adenosine triphosphate (ATP) in insects has suggested that this compound may have as important a function in the cellular metabolism of insects as it is known to have in mammals. Although, with the exception of muscle, the significance of dephosphorylation of ATP in mammalian metabolism is still obscure, the resolution of the mechanism of ATP breakdown has been furthered by the partial characterization of this process in other tissues. Analogously, the first reports of dephosphorylation of ATP in insects were with muscle. No doubt contributory insight into this process can be obtained in insects also by a study of the action of other tissues towards ATP.

Compared with the large amount of information available for mammalian tissues, little is known of adenine nucleotide breakdown in insects. Gilmour (1948) noted a Mg-activated soluble enzyme, derived from locust myosin extracts, which split both labile phosphates from the ATP molecule. A similar observation was made by Barron and Tahmisian (1948) with American cockroach muscle homogenates. The effect of temperature on an apyrase in cockroach muscle was studied by Chin (1951). Recently, Gilmour and Calaby (1952) have reported further investigations on the soluble apyrase found in the muscle of locusts. In conjunction with the confirmation that this enzyme was Mg-activated and removed both labile phosphates from ATP, they demonstrated that although traces of adenylate kinase (myokinase) activity were present in their preparation, this mechanism was not responsible for the removal of the second phosphate. Further, the same apyrase could utilize inosine triphosphate (ITP), adenosine diphosphate (ADP) and inosine diphosphate (IDP) as substrates, but was without effect on adenosine monophosphate (AMP) and several other organic phosphates and pyrophosphates. In addition to the apyrase, it was noted that the locust also possesses a Ca-activated ATPase which is associated with the myosin fraction of the muscle.

In contrast to the situation in orthopteran muscle, Sacktor (1953) found other mechanisms of ATP breakdown in the indirect flight muscles of the house fly. Here it was shown that mitochondria isolated from these flight muscles possesssed a specific ATPase, in that they cleaved only the terminal phosphate from ATP and that ATP was the only phosphorylated compound which released inorganic phosphate. Although liberation of orthophosphate also occurred when ADP was the substrate, it was proved that this activity was due to the presence of a Mg-activated adenylate kinase, which converted ADP to ATP and AMP, and thus provided substrate for the specific ATPase. The mitochondrial ATPase, furthermore, was activated by Mg and Mn but not by Ca ions. On the other hand, an ATPase associated with the muscle *fibrils* was Ca-activated. A soluble fraction of whole thoracic homogenates, like the mitochondria, possessed a Mg-activated ATPase; but this could be distinguished from the mitochondrial ATPase by concurrent inorganic pyrophosphatase activity as well as by differences in the effects of several inhibitors.

The contrast in the mechanism of ATP breakdown between the specialized fibrillar muscle of the house fly and locust muscle leads to the question as to the situation in the other tissues of an insect. Moreover, such investigations should afford a better understanding of the relatively uncomprehended metabolic function of the various insect tissues. In this respect, an approach to a related problem in the comparative physiology of insect tissues was previously reported (Sacktor and Bodenstein, 1952). It was found that different tissues of the American cockroach had different cytochrome *c* oxidase activities which were related to their probable metabolic performance. Furthermore, these results corresponded strikingly with the tracheation of these tissues, as reported by Day (1951). Also, it was discovered that the cytochrome *c* oxidase activity of several tissues was significantly influenced by the sex of the animal. This supplemented the previous report by Barron and Tahmisian of enzymatic differences between male and female muscle homogenates. Accordingly, the present communication is concerned with the relative capabilities of the various cockroach tissues, male and female, to dephosphorylate ATP, and with the influence of bivalent cations on this process. From such findings, further investigations on the mechanism of nucleotide breakdown in insects can be logically approached.

Experimental Procedures

Preparation of tissues. Adult male and female roaches were dissected in 0.9% KCl and the desired tissues were removed as described previously (Sacktor and Bodenstein, 1952). The tissues, immediately prior to enzymatic assay, were homogenized for 30 seconds in 1.0 ml. KCl solution with a Potter-Elvehjem homogenizer. Separate determinations were made on the tissues from each of 10 roaches. Because of the small amount of tissue available from one roach, the separate tissues of two roaches were pooled for the experiments with brain and Malpighian tubes. Thus, 20 roaches were used for the 10 determinations of the enzymatic activity of these tissues.

Determination of enzyme activity. The dephosphorylation of ATP was determined by assaying for inorganic phosphate after 15 minutes of incubation at room temperature (22 to 25 degrees C.). The final concentration of ingredients was: tris (hydroxymethyl) aminomethane buffer, pH 7.4, 0.03 M; ATP (Na salt from Pabst Laboratories) 0.2%; $MgCl_2$, or $CaCl_2$, 10^{-3} M; 0.2 ml. of tissue homogenate (except muscle, where but 0.1 ml. was used); and 0.9% KCl to a final volume of 1.0 ml. Inorganic phosphate was measured by the method of Sumner (1944) in a Klett-Summerson photoelectric colorimeter with a No. 66 filter. Zero time was at the instant of addition of enzyme, and the value determined at this time was subtracted from the final value. With this procedure for enzymatic assay, the rate of dephosphorylation of ATP is directly proportional to the amount of enzyme present and is linear with time during the 15-minute incubation period. A representative set of data is depicted in Figure 1. Furthermore, no inorganic phosphate is liberated either in the absence of homogenate or in the presence of tissue but in the absence of ATP.

Determination of protein. Protein was determined by the method of Lowry *et al.* (1951). Due to the presence in the fat body of urates, which interfere with the color reaction, aliquots of this tissue homogenate were first extracted with acetone and subsequently the protein was precipitated twice with trichloroacetic acid. Crystalline bovine serum albumin was used as the standard protein.

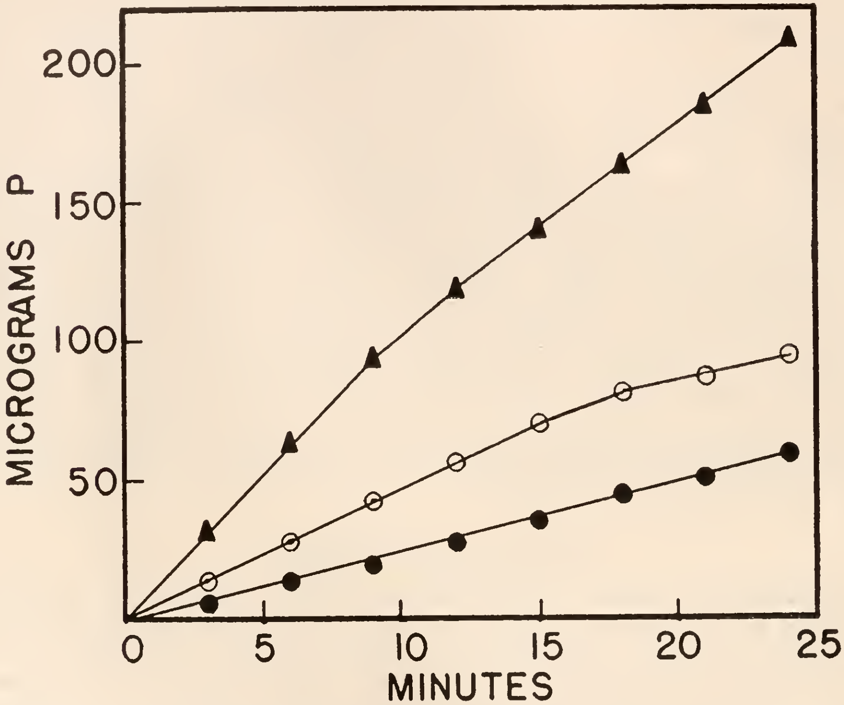

FIGURE 1. The effect of varying concentrations of muscle homogenate on the course of dephosphorylation of ATP. The following symbols: ▲, ○ and ● represent the liberation of inorganic phosphate in the presence of 0.2 ml., 0.1 ml. and 0.05 ml., respectively.

RESULTS

The relative capabilities of various cockroach tissues, male and female, in dephosphorylating ATP, and the influence of bivalent cations on this reaction are shown in Tables I and II.

Dephosphorylation by tissues. The data in Tables I and II reveal that insect tissues differ in their ability to dephosphorylate ATP. In general, based on their activity, they can be grouped into three categories. These are (1) those with greatest activity: muscle, fat body and Malpighian tubes; (2) those with moderate

TABLE I

Dephosphorylation of ATP
Males

Tissue	Control		Calcium		Magnesium	
	micrograms P/hour/mg. protein					
	Mean	± S.E.	Mean	± S.E	Mean	± S.E.
Muscle	119	± 11	237	± 13	516	± 39
Midgut	17	5	29	6	59	10
Foregut	33	6	59	11	77	8
Hindgut	25	5	44	8	103	14
Malpighian tubes	94	21	109	23	145	27
Fat body	140	33	179	44	353	38
Brain	60	10	67	24	113	16
Nerve cord	45	13	54	9	161	25

Control values represent micrograms P liberated in the absence of added bivalent ions. Ca and Mg ion concentration was 10^{-3} *M*. Each datum is the average of 10 determinations.

activity: brain and nerve cord; (3) those of low activity, namely, the three portions of the alimentary canal: hindgut, foregut and midgut. Muscle, as might have been predicted, is the most active. The rate of dephosphorylation by the fat body suggests that this organ may be the site of more extensive cellular metabolism than heretofore realized. This supports recent observations by Bodenstein (1953) on the metabolic role of the fat body. He found that the intermediate metabolism of this tissue responded considerably to changes in the hormonal situation. Although for the most part the gut portions possess relatively little dephosphorylating activity, a notable exception is the hindgut of the female roach.

TABLE II

Dephosphorylation of ATP
Females

Tissue	Control		Calcium		Magnesium	
	micrograms P/hour/mg. protein					
	Mean	± S.E.	Mean	± S.E.	Mean	± S.E.
Muscle	231	± 31	396	± 33	556	± 71
Midgut	20	5	26	6	65	12
Foregut	45	10	68	13	99	22
Hindgut	99	18	111	21	148	27
Malpighian tubes	118	23	143	21	120	19
Fat body	95	26	127	42	159	54
Brain	47	10	64	10	99	17
Nerve cord	74	16	74	17	140	27

Control values represent micrograms P liberated in the absence of added bivalent ions. Ca and Mg ion concentration was 10^{-3} *M*. Each datum is the average of 10 determinations.

Effect of bivalent ions. The data in Tables I and II demonstrate the influence of bivalent cations on ATP dephosphorylation. It is apparent that both Ca and Mg ions stimulate the release of inorganic phosphate from ATP, but that Mg^{++} is more effective. There are, however, differences in the magnitude of response to these ions with the different tissues. Thus, muscle is activated almost 100% by Ca^{++} whereas the slight stimulation observed with brain, nerve cord, Malpighian tubes and fat body was not statistically significant. The Ca-activation noted with the gut parts may represent the effect of this cation on the intrinsic musculature. Mg ions do not have a considerable effect on dephosphorylation of ATP by the Malpighian tubes but, in contrast, do activate the other tissues notably.

Effect of sex. A comparison of Tables I and II indicates that the dephosphorylation of ATP by a given tissue may be influenced by the sex of the roach. Thus, the enzymatic activity of the muscle in the female is significantly (beyond the 0.01 level) greater than that in the male, either in the absence of bivalent cations or in the presence of Ca ions, but not in the presence of Mg ions. The failure to observe a sex difference with muscle activated by Mg ions is in agreement with the results of Gilmour and Calaby, who found that the sex of the locust had no influence on the activity of the Mg-activated soluble apyrase. As in muscle, the hindgut of the female is more active than that of the male in the absence of bivalent cations or when stimulated by Ca ions. On the other hand, the fat body of the male, in the presence of Mg ions, is more active than that of the female. The differences, due to sex, exhibited by the other tissues are not statistically significant.

TABLE III

Inhibition of dephosphorylation of ATP by p-chloromercuribenzoate

Inhibitor conc. (*M*)	Per cent inhibition		
	Control	Calcium	Magnesium
5×10^{-6}	7	13	6
1×10^{-5}	40	28	31
5×10^{-5}	82	73	41
1×10^{-4}	89	85	54
5×10^{-4}	96	96	84
1×10^{-3}	97	97	92
5×10^{-3}	100	100	98
1×10^{-2}	100	100	100

Muscle of the male roach was used in these experiments. Control values represent the per cent inhibition in the absence of added bivalent ions. Ca and Mg ion concentration was 10^{-3} *M*.

Effect of inhibitors. The effect of several known inhibitors of dephosphorylation on the activity of male roach muscle homogenates was ascertained since, in a previous study on the dephosphorylation of ATP by house fly flight muscle (Sacktor, 1953), it was found that azide and p-chloromercuribenzoate inhibited mitochondrial ATPase whereas fluoride did not. Fluoride, however, effected considerable inhibition of a soluble ATPase. In contrast with these observations on house fly flight muscle, it was found in the present study that NaN_3 and NaF at concentrations as high as 10^{-2} *M* are without appreciable effect on the dephosphorylation of ATP by roach muscle. However, p-chloromercuribenzoate inhibits dephosphorylation here also, as shown in Table III. A similar effect of this -SH inhibitor was noted by Gilmour and Calaby with their enzyme preparation from the locust.

A comparison of the present results with those obtained with the house fly (Sacktor, 1953) indicates that p-chloromercuribenzoate is approximately 100 times as effective an inhibitor in the roach. The data suggest further that inhibition in the roach is somewhat lessened in the presence of Mg ions. With rat liver mitochondrial ATPase, however, Novikoff *et al.* (1952) found no difference in the inhibition by p-chloromercuribenzoate (5×10^{-4} M) in the presence of Mg or Ca ions.

Discussion

The data presented show that various cockroach tissues differ in their ability to dephosphorylate ATP and that both Ca and Mg ions activate dephosphorylation. The magnitude of stimulation by these cations depends on the particular tissue. It should be emphasized, however, that these results were obtained with the use of a standard experimental procedure for all tissues. This procedure, which is based on optimal conditions for muscle, need not represent the ideal situation for the other tissues.

Complete characterization of the dephosphorylating mechanisms with the various tissues must await further experimentation; nevertheless, an interesting comparison between the enzymatic activities found in roach muscle and in vertebrate muscle is now possible. In a recent study of the biochemical differences between red and white muscle, Lawrie (1952) found that red muscle had more myoglobin and a higher activity of cytochrome *c* oxidase, succinic dehydrogenase and succinoxidase than did white muscle, but, conversely, a lower ATPase activivty. The coloration of red muscle was attributed to the greater content of myoglobin. In the roach, however, the muscle of the male is reddish in color, whereas that of the female is much lighter, almost white (Sacktor and Bodenstein, 1952; Edwards, 1953). It was found, too, that these color variations were reflected in the cytochrome *c* oxidase (Sacktor and Bodenstein, 1952) as well as succinic-cytochrome *c* reductase activities (Sacktor, unpublished data); for, muscles with more coloration had a higher activity; and, as with vertebrate muscle, the dephosphorylation of ATP in female (white) muscle was greater. Furthermore, Barron and Tahmisian (1948) reported more cytochrome *c* and Fe in the male roach muscle. Contrary to the situation in vertebrates, there is no evidence for the presence of myoglobin in roach muscle. These facts indicate that in insects, at least, the color differences may be due to differences in the cytochrome content, and suggest that cytochromes as well as myoglobin are possibly concerned in the color of vertebrate muscle.

Despite many similarities between insect and vertebrate muscle, distinctions can be made even between the various insect species. Differences in the mechanism of ATP breakdown by house fly flight muscle and locust muscle have been described above. In addition, dissimilarities in the action of several inhibitors of dephosphorylation were found between roach muscle homogenates and house fly flight muscle preparations. In the roach muscle, p-chloromercuribenzoate inhibited dephosphorylation whereas azide and fluoride did not. In contrast, with mitochondria isolated from the specialized flight muscles of the fly, ATPase was inhibited by azide as well as by p-chloromercuribenzoate (Sacktor, 1953). Furthermore, fluoride prevented orthophosphate liberation by a soluble fraction obtained from these specialized fibrillar muscles. Other species-specific enzymatic characteristics have been noted recently in other insects. Metcalf and March (1949) found that certain organic phosphates inhibited fly brain cholinesterase but had little effect on bee brain cholinesterase. Supplemental evidence on species variations was obtained by Rockstein and Levine (1951) in their studies on acid phosphatases in five insect species. From these numerous heterogeneities, it is becoming increasingly evident that generalizations on the enzymatic properties of all insects are no longer valid. Careful examination of such parallels and differences in insects may well yield fruitful leads for investigation in the tissues of other animals.

SUMMARY

1. The dephosphorylation of ATP by various tissues of the American cockroach was investigated. It was found that these tissues differ in their dephosphorylating activity and may be rated, in decreasing order, approximately as follows: muscle, fat body, Malpighian tubes, nerve cord, brain, hindgut, foregut and midgut.

2. In general, both Mg and Ca ions activated the breakdown of ATP, although Mg was more effective. The magnitude of activation by these bivalent cations depended on the tissue.

3. Some differences due to sex were found. The dephosphorylating activity of the muscle and hindgut of the female was significantly greater than that in the male either in the absence of bivalent cations or in the presence of Ca ions. The fat body of the male, in the presence of Mg ions, was more active than that of the female.

4. The dephosphorylation of ATP by roach muscle was inhibited by p-chloromercuribenzoate, but not by azide and fluoride. Since these three compounds were effective inhibitors of house fly flight muscle ATPase, this distinction further emphasizes the heterogeneity of ATP breakdown mechanisms in insects.

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PHYSIOLOGY OF INSECT DIAPAUSE. V. ASSAY OF THE GROWTH AND DIFFERENTIATION HORMONE OF LEPIDOPTERA BY THE METHOD OF TISSUE CULTURE

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The metamorphosis of insects, like the growth and maturation of vertebrates, is under the control of an endocrine *system.* In the Lepidoptera, the Diptera, the Hemiptera, and the Blattoidea, this system is known to include a minimum of three endocrine tissues—the brain, the prothoracic glands, and the corpora allata. The *brain hormone* is the secretory product of certain highly specialized neurones in the cerebral ganglion; its principal target appears to be the prothoracic glands. Under the tropic stimulation of the brain hormone the prothoracic glands secrete the *growth and differentiation hormone.* And it is apparently this prothoracic gland hormone which reacts with the tissues to promote growth, molting, and metamorphosis (Williams, 1952a). Within the immature insect, there is yet a third hormone whose source is the corpora allata. The corpus allatum hormone is a conservative factor—a *status quo hormone*—which inhibits metamorphosis by modifying the response of the immature tissues to the growth and differentiation hormone (Williams, 1952b).

Though all these conclusions are based on substantial evidence, it is worth recalling that neither the brain hormone, nor the growth and differentiation hormone, nor the *status quo* hormone has ever been demonstrated by any method of chemical or *in vitro* assay. Consequently, it has been necessary to judge the hormonal action by recourse to such indirect methods as the extirpation and implantation of living endocrine organs, parabiosis, and similar procedures. Indeed, none of the above-mentioned hormonal effects is duplicated when one removes blood from an insect and injects it into a test animal. This fact is scarcely mentioned in the literature (Schürfeld, 1935; Plagge, 1938), though it is a reasonable presumption that such experiments have been carried out by most investigators. In studies of the Cecropia silkworm, we have failed in repeated attempts to demonstrate endocrine activity by the injection of blood. Thus, in numerous experiments performed on brainless diapausing pupae, two-thirds of the blood was drained and replaced by that of mature larvae or developing adults. We have continued this procedure on individual pupae at weekly intervals from one to seven times. In no instance did the transfused blood have any detectable effects on the test animal.

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Experiments of this type have been the principal basis for the view that the hormones of insects are conveyed in the tissues rather than in the blood. If the blood has no demonstrable endocrine activity, then perhaps the hormones of insects, like the auxins of plants (Thimann, 1952), may be transported from cell to cell rather than in the "sap."

With the exception of the blood-borne factor which promotes puparium formation in the higher Diptera (Fraenkel, 1935), the only substantial evidence that hormones concerned with growth and metamorphosis are present in the blood has hitherto been derived from parabiosis experiments. Thus, when two insects are grafted together so that they share the same blood, the one can supply hormones to the other. However, under further analysis, the two animals are found to grow together and attain organic continuity before the hormonal effect is manifested (Bodenstein, 1938; Williams, 1946). Moreover, this tissue union is established even when glass or plastic tubes have been interposed between the animals (Williams, 1947; Lüscher, 1948). The parabiosis experiments have therefore intensified rather than resolved the problem of the mode of transport of insect hormones.

The central and persistent difficulty has been the lack of any practical method of assaying the insect hormones. In the absence of such tests, it has been impossible to detect the hormones in the blood or in the tissues. It has also been impossible to proceed with their extraction, purification, and chemical characterization.

The present study was directed toward the development of an assay for the growth and differentiation hormone. For reasons considered above, it was obvious that the intact insect failed to provide a basis for such a test. Attention was therefore focussed on the culture of an individual tissue in the hope that an *in vitro* system might furnish a method of biological assay.

Practical considerations directed the choice of an appropriate tissue. The latter should be readily available and in a uniform state at the outset. It should undergo prompt response at the beginning of adult development; *i.e.,* during the period when the action of the growth and differentiation hormone is uncomplicated by the simultaneous presence of the *status quo* hormone. Moreover, its response to the growth and differentiation hormone should be accompanied by conspicuous change. Finally, the tissue should be simple to culture.

One tissue above all others appeared to satisfy these requirements; namely, the male sex cells of the diapausing pupal testes—a cell type which at the outset of adult development undergoes prompt and spectacular transformation into spermatozoa. Could such cells be cultured *in vitro?* Assistance in so doing was sought in a survey of the scattered literature.

In Vitro Culture of Insect Tissues

The first attempt to culture insect tissues was that of Goldschmidt (1915, 1916, 1917). Working in Harrison's laboratory, Goldschmidt cultured the male sex cells of Cecropia pupae and observed definite maturation in simple hanging-drop preparations. Unfortunately, no record was made of the exact status of the "pupae" that served as donors of blood; consequently, no endocrinological significance was apparent in Goldschmidt's results.

Similar hanging-drop preparations have subsequently been used to observe meiosis in the male germ cells of adult Orthoptera and Hemiptera (Lewis and

Robertson, 1916; Bêlâr, 1929; Baumgartner and Payne, 1930; Ris, 1949). But in all these instances the germ cells were observed for only a few hours after their isolation, and the development *in vitro* was clearly a continuation of processes already underway in the intact insect.

The only direct support for the view that the *in vitro* growth of insect tissues may depend on hormonal factors is an isolated study reported twenty-five years ago. In experiments designed to solve a morphological problem, Frew (1928) observed that the leg discs of blowfly larvae evaginated and underwent definite development in a culture medium prepared from early pupae, but failed to do so in a medium prepared from older pupae or from larvae. However, Frew's experiments are difficult to interpret since the data are presented in meager detail and technical difficulties are emphasized.

Numerous observations have been made of the behavior of cultured blood cells. In one of the early studies in this field, Glaser (1917) found that the blood cells of caterpillars and adult grasshoppers underwent limited multiplication in simple cultures, either in blood or in Locke's solution. However, several other investigators have been unable to detect mitotic activity in hanging-drop preparations of blood. Goldschmidt (1916) observed only amitotic activity as the blood cells of Cecropia formed a syncytial tissue in the cultures. Similar *in vitro* formations of blood cell syncytia have been observed in *Oryctes* (Lazarenko, 1925), *Periplaneta* (Taylor, 1935; Trager, 1935), larval *Bombyx mori,* and pupal *Platysamia cecropia* (Trager, 1935). Fischer and Gottschewski (1939) report the multiplication of *Drosophila* blood cells in an artificial medium which is not described in detail; growth was also observed in isolated leg and wing anlagen.

But the continuous multiplication of cells, comparable to that obtained in cultures of vertebrate tissues, has apparently not been achieved on insect material. The nearest approach was Trager's (1935) finding that certain cells in the ovary of mature silkworm larvae underwent considerable proliferation when cultured in a medium containing a small quantity of larval blood. Moreover, Charin (1930) reports the migration and limited multiplication of certain lepidopterous tissues *in vitro*—a finding reminiscent of the so-called "residual growth" of vertebrate tissues in the absence of embryonic extract. In the remaining instances in which insect tissues have been successfully explanted (Murray, 1926; Gottschewski and Fischer, 1939; Stern, 1940), residual activity was apparent, but no actual growth occurred.

From this survey of the literature, it is apparent that the cultivation of insect tissues has not been a widely used or highly successful technique. In view of the fact that morphogenetic activity in the insect itself is intermittent and evidently related to growth-controlling hormones, the scanty success achieved in the culture of insect tissues suggests that these same endocrine factors may be required for the growth of isolated tissues.

DEVELOPMENT OF SEX CELLS IN THE INTACT INSECT

The testes of the Cecropia silkworm, at all stages, are divided internally into four tubules by three septa radiating from the region of the vas deferens. The tubules contain the germ cells, the majority of which are gathered into small balls and enclosed by an envelope of flattened cells—the whole forming the so-called "germinal cysts" (Dederer, 1907; Omura, 1936). The latter are either free in the fluid which fills the testicular tubules or suspended in grape-like clusters from minute tracheoles. The maturity of the germ cells can be judged by their size, by the number in each cyst, and by the fact that their early development transforms the cysts into hollow spheres.

During the prepupal period the cells in a few of the more mature cysts undergo one or both maturation divisions, all of the cells in a given cyst dividing simultaneously. In a small proportion of cysts, spermatids begin to differentiate at this time—a process invariably accompanied by considerable elongation of the cyst as a whole. Cells which undergo this precocious maturation during the prepupal period appear to die shortly thereafter. Thus the testes of diapausing pupae include cysts containing degenerate cells with pycnotic nuclei, but no recognizeable spermatids. Consequently, the germ cells in the pupal testes closely resemble those present in mature larvae and range from spermatogonia to primary spermatocytes.

During the pupal diapause there is no indication of further development in any of these cells. However, immediately after the termination of diapause and the initiation of adult development, a rapid and impressive maturation takes place. Mitotic divisions occur in the immature cysts containing spermatogonia, while meiotic divisions occur in the more mature cysts containing primary spermatocytes. These changes are synchronized with the first signs of adult development in the pupal hypodermis. Shortly thereafter, even the immature germ cells, comparable to those commonly observed in larvae midway the fifth instar, promptly transform into spermatids. By the end of the first week of adult development, nearly all the sex cells in the testes have been converted to spermatids.

Materials and Methods

Sex cells from the testes of diapausing pupae of *Platysamia cecropia* and *Samia walkeri* (*cynthia*) were cultured in the blood of larvae, diapausing pupae, developing adults, and adults of the same species. The cultures were prepared as follows:

A male diapausing pupa was selected and surface-sterilized by immersion in a solution of 0.05% mercuric chloride in 50% ethyl alcohol, followed by thorough rinsing in sterile distilled water—a treatment that had no detectable effects on the insects at any stage in development. A transverse slit was then cut in the dorsal region between the fourth and fifth abdominal segments and, with slight pressure on the abdomen, the testes were caused to herniate. Each testis was then transferred to a depression slide containing blood previously collected from a suitable donor.

Rupture of the testicular envelope liberated thousands of germinal cysts into the small pool of blood. By means of a fine pipette a drop of the resulting suspension was placed on the underside of a coverslip and the latter sealed to a depression slide with melted paraffin. It was impossible to pipette a uniform suspension of cysts, and the number included in each culture varied considerably. Ordinarily, each preparation contained at least 75 cysts—indeed, a relatively large number seemed to favor the survival of the culture. Five or six cultures were usually prepared from each sample of blood. Each culture was immediately examined under the compound microscope for any indication of development; it was then placed at the constant temperature of 25° C. or 30° C. and examined at daily intervals.

The blood used as the culture medium was obtained by slicing the cuticle and underlying hypodermis from the facial region of donor insects and draining the blood into a depression slide containing a few small crystals of phenylthiourea (twice recrystallized from the Eastman product). The blood of these silkworms, like that of most insects, undergoes rapid darkening unless the activity of tyrosinase is inhibited. This darkening results from the enzymatic oxidation of phenols to quinones. Such darkened blood is wholly unsuitable as a culture medium and, in fact, is poisonous. Phenylthiourea specifically inhibits tyrosinase and opposes these changes without, in itself, being toxic. It was the use of this chemical that made the present experiments technically possible.

Blood from developing adults contains a large amount of cellular debris from disintegrating fat body. This was largely eliminated by allowing the particulate matter to settle and withdrawing the relatively clear blood with a pipette. In a number of experiments, the blood was centrifuged to remove all cells—a procedure which seemed to be inconsequential.

The preparation of cultures was accomplished with sterile instruments and glassware; the technique was simple and sufficiently rapid to make elaborate precautions against air-borne organisms unnecessary. Approximately 2% of the cultures eventually showed contamination with microorganisms. Since a number of identical cultures were always prepared, contamination was not a major source of difficulty.

Pupal Germ Cells Cultured in Blood of Pupae, Developing Adults, and Adults

The results of 179 successful cultures prepared in the blood of post-larval donors are summarized in the lower portion of Table I. This total includes all cultures in which any development was detected, though in some instances the cultures survived a relatively short time. Since the onset of development was occasionally delayed until the fourth day, the absence of development was not considered significant until the fourth day; cultures showing no development but failing to survive four days are not included in the data. The death of germ cells *in vitro* seemed to be closely followed or even preceded by visible degenerative changes, and it has been assumed that the cells were alive until these changes appeared.

1. In blood of diapausing and previously chilled pupae

As indicated in Table I, germ cells in the majority of cultures prepared in the blood of pupae which had been in the dormant state for 5 to 16 weeks retained their original configuration (Fig. 1A) and showed no development whatsoever, notwithstanding the fact that many of the cultures survived for a week or longer. In certain preparations the meiotic divisions were evident in an occasional cyst (Fig. 1B). However, the cells in such cysts underwent degeneration within two or three days without forming elongated spermatids, and development was initiated in no additional cysts. On rare occasions, cysts like those shown in Figures 1C and 1D were detected in cultures several days old. Cultures showing any of these patterns of minimal response have been listed as undergoing "slight development."

Results identical to those obtained in the blood of dormant pupae were observed in cultures prepared in the blood of previously chilled pupae—that is, pupae which had been stored at 5° C. for a sufficient period to assure the initiation of development after about two weeks' exposure to 25° C. Altogether it is evident that germ

TABLE I

Pupal germ cells cultured in blood from larvae, prepupae, pupae, and developing adults

Source of blood	Age of donors (days)	No. of donors	No. of cultures	Development in cultures		
				None	Slight	Progressive
Mature 5th instar larvae, not yet spinning	0	3	15	14	1	0
Larvae, spinning outer capsule of cocoon	0–1	3	10	8	2*	0
Larvae, spinning inner capsule of cocoon	1–2	5	34	5	0	29
Larvae, finished spinning cocoon	2–5	3	9	0	3*	6
Green prepupae	5–8	3	11	0	1*	10
White prepupae	8–9	2	10	0	4*	6
Fresh pupae	9–10	2	13	0	2*	11
Diapausing pupae	After 3 weeks at 25° C.	4	17	2	9	6
Diapausing pupae	After 5 to 16 weeks at 25° C.	13	40	26	14**	0
Chilled diapausing pupae	After 12 to 35 weeks at 5° C.	7	23	20	3*	0
Developing adults	1st to 5th day	15	47	0	6*	41
Developing adults	7th to 18th day	6	18	0	2*	16

* All cultures died within 48 hours after development was initiated.
** Six cultures died within 48 hours after development was initiated.

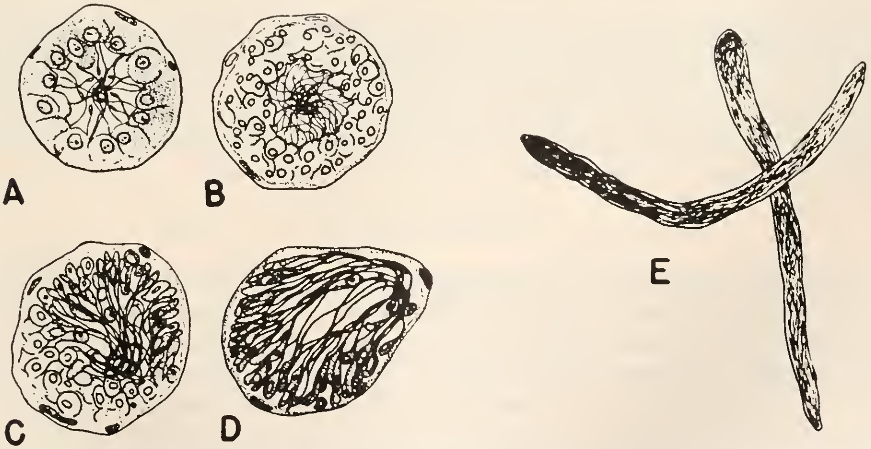

FIGURE 1. Characteristic stages in the development of pupal Cecropia sex cells in hanging-drop cultures of active blood. *A* is an optical section of a testicular cyst from a diapausing pupa; the enclosed cells are primary spermatocytes. *B* shows a typical cyst after several days of culture; the sex cells have just completed meiosis. Development proceeds in *C* to yield early spermatids within the still spherical cyst. In *D* the differentiation of spermatids has continued and the cyst as a whole has begun to elongate. *E* records the final stage of *in vitro* differentiation; the cysts are greatly elongated and contain well-differentiated spermatids. *A* through *D*, × 250; *E*, × 140.

cells are incapable of undergoing rapid or extensive development in the blood of diapausing or previously chilled pupae.

2. *In blood of developing adults and adults*

With the termination of pupal diapause and the initiation of adult development, a remarkable change was evident in the blood's capacity to promote the development of sex cells. Within 24 to 48 hours after cultures were prepared in the blood of developing adults, the cells in one or more cysts ordinarily showed the completion of the maturation divisions. Within the following 24 hours, meiosis usually occurred in many additional cysts. Meanwhile, the more precocious cysts underwent elongation, the nuclei moving to one pole and the axial filaments of the developing spermatids growing towards the other. At this stage a large proportion of the cysts corresponded to those diagrammed in Figures 1B to 1D. After a week in culture, the majority of cysts showed definite elongation; many were ten to fifteen times longer than wide and contained well differentiated spermatids (Fig. 1E).

Since the germ cells usually failed to survive longer than a week in culture and not infrequently died sooner, the final degree of differentiation was less impressive in those cultures where development was delayed or proceeded slowly. Characteristically, however, continuous progress was made toward the spermatid stage as long as the germ cells survived. Accordingly, "progressive development" was distinguished, not so much by the absolute amount of differentiation, as by its sustained progress. The results in Table I show that progressive development occurred in blood obtained from animals after the initiation of adult development.

3. *Germ cells of Platysamia cecropia cultured in the blood of Samia walkeri*

Cultures prepared from blood and testicular tissues of *Samia walkeri* showed the same behavior as those prepared from *Platysamia cecropia*. Moreover, the sex cells of *Platysamia* developed as promptly in the post-pupal blood of *Samia* as in the post-pupal blood of *Platysamia* itself. Progressive development also occurred in experiments of the reverse type; that is, when the germ cells of *Samia* pupae were cultured in blood obtained from developing adults of *Platysamia*.

Blood Cells and Spermatogonia In Vitro

In cultures prepared in uncentrifuged blood, many of the blood cells flattened themselves against the coverslip and within two days coalesced to form small areas of syncytial tissue. Little further change occurred when the blood cells of diapausing pupae were cultured in the blood of diapausing pupae, though the cells appeared to survive for over a month. In contrast, in cultures prepared in the blood of developing adults, a slow increase in the size and complexity of the blood cell syncytia became evident after five or six days. In cultures maintained for two weeks or longer and subsequently fixed and stained, scattered mitotic figures were evident in the flattened blood cells.

In cultures prepared in the blood of early developing adults and maintained for longer than the usual period of one week, masses of small cells slowly appeared. These cells failed to correspond to any of the various types of blood cells, nor did they show the latter's tendency to flatten and form syncytial tissues. Inspection of fixed and stained cultures revealed frequent mitotic figures.

Pupal testes invariably contain a small number of cells not yet enclosed within cysts. These cells, which are presumed to correspond to early spermatogonia, are inevitably released along with the cysts of late spermatogonia and spermatocytes when the testes are ruptured in the preparation of the cultures. The cells observed to multiply in cultures closely resembled the early spermatogonial cells in terms of the presence of a scanty, dense cytoplasm and a large vesicular nucleus.

It thus appears that the property of the blood of developing adults which promotes the differentiation of spermatocytes also stimulates cells of a different character, such as blood cells and spermatogonia, to divide. Consequently, it can truly be said that the active agent in the blood of developing adults promotes both growth and differentiation.

Pupal Sex Cells Cultured in Larval Blood

Observations were made of the differentiation of pupal cells when cultured in larval blood. The preparation of such cultures was at first complicated by a jelly-like clotting of the blood when withdrawn from caterpillars. Fortunately, this viscous change, due apparently to adhesions between blood cells, disappeared when the blood was stored for 12 to 24 hours at 3° C. Under this circumstance, the cells formed dense clumps and the clear fluid residue could once more be used in culturing the pupal spermatocytes.

The results of cultures of pupal cysts in larval blood (Table I) indicate that the blood of mature fifth instar larvae is essentially inactive until a stage signalled by the spinning of the inner coat of the cocoon. The larval blood then becomes active in promoting the transformation of pupal sex cells. From these results, it appears that a factor required for the maturation of pupal cells increases markedly in concentration, not only at the outset of adult development, but also just prior to pupation. This factor, as indicated in Table I, persists in the blood throughout the prepupal period and then slowly falls to sub-threshold concentration following pupation.

Sex Cells Cultured in Diluted Blood and in Mixtures of Active and Inactive Blood

The failure of the blood of diapausing pupae to support the maturation of sex cells might be attributed either to the presence of an inhibitory factor, or to the absence of a necessary growth hormone. To distinguish between these possibilities, cultures were prepared in mixtures of "dormant" blood and "active" blood and in blood diluted with insect Ringer's solution (Ephrussi and Beadle, 1936).

As recorded in Table II, the low activity of dormant blood was not altered by the addition of an equal volume of Ringer's solution. Hence, if the diapausing blood contains an inhibitory substance, the latter is not rendered sub-threshold by this degree of dilution. Table II also shows that active blood remained active after the addition of an equal volume of dormant blood. These findings indicate that the blood of developing adults contains a growth-promoting hormone which is in far lower titer in the blood of mature diapausing pupae.

TABLE II

Pupal germ cells cultured in mixtures of bloods and in bloods diluted with Ringer's solution

Culture medium	Number of experiments	Number of cultures	Development in cultures		
			None	Slight	Progressive
Blood from dormant pupae plus Ringer's solution; equal parts	4	12	8	4*	0
Blood from developing adults plus Ringer's solution; equal parts	5	17	0	0	17
Blood from developing adults and dormant pupae; equal parts	5	22	0	12**	10

* All cultures died within 48 hours after development was initiated.

** Seven cultures died within 48 hours.

PROPERTIES OF THE GROWTH-PROMOTING SUBSTANCE PRESENT IN ACTIVE BLOOD

The nature and properties of the growth-promoting hormone in the blood of pupating larvae and developing adults were studied by subjecting active blood to various treatments and then re-testing its capacity to promote development *in vitro*.

1. *Effects of storage at 3° C. and of freezing*

Hormonal activity was retained when active blood was stored under refrigeration. As previously mentioned, blood collected from pupating larvae was routinely stored at 3° C. for 12 to 24 hours without loss of activity. Indeed, in two experiments involving a total of eight cultures, progressive development was observed in prepupal blood previously stored at 3° C. for four days. In another experiment, blood obtained from developing adults was frozen at − 29° C., then thawed at room temperature, then re-frozen and re-thawed. When tested in six cultures, full activity was observed—a finding which demonstrates that the hormone is stable at low temperatures.

2. *Effects of heat treatment*

Abrupt heating to temperatures above 60° C. caused the blood to coagulate in a solid mass. This difficulty was avoided by raising the temperature in steps of approximately 5°, and removing the precipitate by centrifugation after each step. By this procedure a small fraction of clear supernatant could be obtained even after heating the blood to 100° C.

When active blood was heated to 56° C., a scanty white precipitate formed; after centrifugation the supernatant proved suitable for use in the cultures. But when the temperature was raised to 60° C., a copious white precipitate appeared; the supernatant, when tested, now caused extreme fragmentation and granulation of the sex cells and death within a few hours—a result which we attribute to the altered osmotic pressure of the blood. Consequently, in order to test blood previously heated to temperatures above 60° C., it was necessary to mix the supernatant with an equal volume of untreated, inactive blood obtained from diapausing pupae.

When prepared in this manner, the culture medium regained its compatibility with the germ cells. In each such experiment the inactivity of the unheated diapausing blood was verified in control cultures.

Experiments testing the growth of spermatocytes in initially active blood are summarized in Table III. Pupal germ cells showed prompt and progressive development to spermatids when cultured in blood previously heated for fifteen minutes at 75° C. or at any lower temperature. In contrast, the behavior of cultures prepared with blood heated for ten minutes at 80° C. was indicative of a definite decrease in growth-promoting activity. Though three of four such cultures showed slight development after three days, no further development occurred, notwithstanding the fact that the cells survived for approximately seven days. The fourth culture likewise appeared to survive for about a week, but showed no development whatsoever. A similar negative result was observed in blood previously heated to 100° C. for ten minutes, though in three cultures the cells survived for at least six days. In summary, these results indicate that the blood's hormone is stable and unprecipitated by brief exposure to a temperature of 75° C., but is progressively destroyed or precipitated at higher temperatures.

TABLE III

Pupal germ cells cultured in media containing active blood previously heated to various temperatures

Preliminary heat treatment		Number of satisfactory cultures	Development in cultures
Temp. (° C.)	Time (min.)		
56	25	10	Progressive
65*	15	7	Progressive
70*	15	6	Progressive
75*	15	5	Progressive
80*	10	4	Slight
100*	10	3	None

* Supernatant diluted with equal parts of inactive blood.

3. *Effect of dialysis*

Approximately one ml. of active blood was placed in a viscous casing and dialyzed for 24 hours at 1° C. against 100 ml. of insect Ringer's solution. The undialyzed fraction, mixed with an equal volume of blood from dormant pupae, was tested in five cultures and found to retain its capacity to promote the development of pupal germ cells. The experiment was repeated, increasing the time of dialysis to two days. The undialyzed fraction, mixed with an equal volume of dormant blood, caused prompt development when tested. Evidently, the hormone is either a large molecule or is closely bound to such a non-dialyzable component.

DISCUSSION

The tissue culture studies demonstrate that the blood of the Cecropia silkworm contains a growth factor whose concentration or activity undergoes systematic change during the larval-pupal-adult transformation. Its initial low titer in the blood of the mature larva increases markedly just prior to the prepupal stage; *i.e.,* during the period when the growth and differentiation hormone induces the larva to pupate (Williams, 1952a). Within the newly formed pupa the factor then persists in gradually diminishing titer, only a trace being encountered in the blood of the diapausing pupa several weeks later.

For months thereafter the blood of the diapausing pupa remains virtually inactive. But just prior to the termination of diapause and during the ensuing first week of adult development the factor reappears in high titer—a change which correlates precisely with the period of action of the prothoracic glands (Williams, 1952a). For these several reasons we are persuaded that the factor which promotes the *in vitro* development of the sex cells is the same growth and differentiation hormone which, within the intact insect, is required for the growth and maturation of the gonads and of all other tissues (Williams, 1952b).

Efforts were made to demonstrate this fact by more direct procedures; namely, by the implantation of active prothoracic glands into cultures prepared in inactive blood. Experiments of this type were complicated by bacterial contamination of the fragments of the prothoracic glands during their dissection from developing adults. Eventually, three hanging-drop cultures were prepared containing both germinal cysts and fragments of living prothoracic glands. Though the sex cells and the glandular cells appeared to survive for several days, no development took place. Similar negative results were obtained in cultures fortified with extracts of active prothoracic glands in saline or in inactive blood.

The apparent failure of the prothoracic glands to continue their secretory activity under the highly artificial conditions existing in hanging-drop cultures is not surprising, particularly in view of Fukuda's (1944) finding that a normal oxygen supply is necessary for the secretory activity of the prothoracic glands—a condition obviously not met in small, sealed cultures.

If one accepts the tissue culture test as a valid assay for the growth and differentiation hormone, then it is clear that the isolated testicular tissue is far more sensitive to the hormone than are the intact testes or the insect as a whole. The isolated germ cells respond to concentrations of growth and differentiation hormone that are sub-threshold within the intact insect. Thus the sex cells developed promptly when cultured in the blood of recently pupated animals in which all development had ceased (Table I). Indeed, the blood of pupae which had been dormant for months not infrequently caused a slight but definite response of the cultured cells. Moreover, it is worth recalling that during the second week of adult development the blood showed considerable hormonal activity, notwithstanding the fact that the prothoracic glands, the source of the hormone, are known to undergo complete degeneration during the first week of adult development. While attesting to the stability of the growth and differentiation hormone within the insect, this finding suggests the further possibility that the hormone may continue to influence the development of the insect during periods when the prothoracic glands are inactive or even absent, as, for example, during the maturation of the eggs.

There is clear evidence that one reason for the high sensitivity of the *in vitro* procedure is the absence of the testicular envelopes which are interposed between the sex cells and the blood within the intact insect. During the period of prepupal development this barrier apparently shields the germ cells from the threshold concentration of hormone in the surrounding blood. For if the sex cells are removed from the testes of prepupae and cultured in the blood of the very same animals, normal spermatogenesis is observed. And, as we have seen, the same is true within the newly formed pupa where the concentration of hormone is sufficient to cause spermatogenesis *in vitro* but not *in vivo*.

The sensitive response of cultured germ cells affords the first direct evidence that the growth and differentiation hormone of insects is, indeed, a blood-borne hormone; it also provides a means of recognizing this factor. The fact that the germ cells of either *Platysamia cecropia* or *Samia walkeri* develop in the blood of the opposite species demonstrates that the growth and differentiation hormone of Lepidoptera is, to this degree, neither species- nor genus-specific. The hormone also appears to be non-dialyzable and destroyed or precipitated by exposure to temperatures above 75° C.—properties which suggest that the growth and differentiation hormone is either a protein or a smaller molecule tightly conjugated to a protein. Though the tissue culture technique is only roughly quantitative in its present form, the growth and differentiation hormone, according to the evidence already at hand, appears to be relatively stable within the insect and to wax and wane in synchrony with the recurrent bouts of secretory activity by the prothoracic glands.

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SUMMARY

1. Spermatogonia and spermatocytes isolated from the testes of dormant pupae of the silkworms, *Platysamia cecropia* and *Samia walkeri* (*cynthia*), promptly develop into well differentiated spermatids when cultured in hanging-drops of blood obtained from pupating larvae or developing adults of these species. Comparable development does not occur in cultures prepared in blood obtained from diapausing pupae or from mature larvae prior to a well-defined period before the onset of the prepupal stage.

2. The *in vitro* development of the sex cells signals the presence of a growth-promoting hormone. In inactive blood, as during the pupal diapause, this hormone is ordinarily in sub-threshold titer.

3. In addition to promoting the maturation of spermatocytes, the hormone causes the *in vitro* multiplication of blood cells and spermatogonia.

4. Pupal blood is active in promoting the development of germ cells *in vitro* after the initiation of adult development; the blood of mature larvae becomes active at a stage signalled by the spinning of the inner coat of the cocoon. This timing corresponds precisely with the periods during which the prothoracic glands secrete the growth and differentiation hormone which provokes the pupation of the larvae and the adult development of the pupa.

5. Evidence is presented that the development of the germ cells *in vitro* reflects the presence in the culture medium of the growth and differentiation hormone secreted by the prothoracic glands. The development of the sex cells in the tissue culture appears to be an exceptionally sensitive test for this hormone.

6. By the use of this test the hormone was found to be non-dialyzable and destroyed or precipitated by exposure to temperatures higher than 75° C. Accordingly, it is concluded that the growth and differentiation hormone is either a protein or a smaller molecule conjugated to a protein.

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THE PHYSIOLOGY OF INSECT DIAPAUSE. VI. EFFECTS OF TEMPERATURE, OXYGEN TENSION, AND METABOLIC INHIBITORS ON *IN VITRO* SPERMATOGENESIS IN THE CECROPIA SILKWORM[1]

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Throughout the previous papers of this series of studies of the Cecropia silkworm it has been necessary to infer the metabolic reactions of the insect's tissues from the effects of various agents and experimental conditions on the whole or subdivided organism. Manifestly, it would be highly desirable to test these findings on an isolated tissue. Fortunately, this objective is now accessible by virtue of the successful *in vitro* culture of the male sex cells, as demonstrated by Schmidt and Williams (1953). As these investigators have shown, the pupal spermatocytes of the insect, when cultured in simple hanging-drop preparations, undergo meiosis followed by a remarkable differentiation into spermatids and spermatozoa.

In the present investigation we have attempted to define the effects of temperature, oxygen tension, and certain metabolic inhibitors on *in vitro* spermatogenesis. Agents were selected for study whose actions on intermediary metabolism had previously been defined.

MATERIALS AND METHODS

1. Culture methods

The methods utilized in culturing the spermatocytes were modifications of those described by Schmidt and Williams (1953). In order to block the tyrosine-tyrosinase reaction, several crystals of phenylthiourea (twice recrystallized from the Eastman product) were added to each sample of blood. Pairs of testes were removed from either diapausing or brainless diapausing male pupae and placed in a depression slide containing several drops of hormone-containing blood from a Cecropia pupa that had just initiated adult development. Each testis was then torn open, releasing thousands of spermatocytal cysts into the blood. The resulting suspension was transferred to a sterile test tube and additional active blood added to a volume of two ml. Each such suspension contained about 100 to 300 cysts per drop. The tubes were then plugged with cotton and stored at 2° C. until used. While failing to develop at this low temperature, the cysts remained viable and, at the convenience of the investigator, could be used in experiments during the following week. Individual hanging-drop cultures were prepared by pipetting a

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drop of cyst suspension, about 20 mm.^{3} in volume, onto the underside of a coverslip; the latter was then sealed to a depression slide with melted paraffin.

In experiments where numerous cultures were required, the procedure was altered, as follows. Glass plates, 5″ × 5″ × 0.06″, were covered with thin films of petrolatum and sterilized. "Sitting-drops" of about 20 mm.^{3} volume were pipetted in rows on each plate. A brass ring, 4″ in diameter × 0.125″ wide × 0.08″ thick, was placed on the glass plate surrounding the drops, and another 5″ × 5″ × 0.06″ glass plate set on top of the ring. The chamber, thus formed, was sealed with melted paraffin. In experiments in which cysts were exposed to specific gaseous environments, the culture chambers were modified by the addition of inlet and outlet tubes to permit the circulation of suitable gas mixtures (Fig. 1).

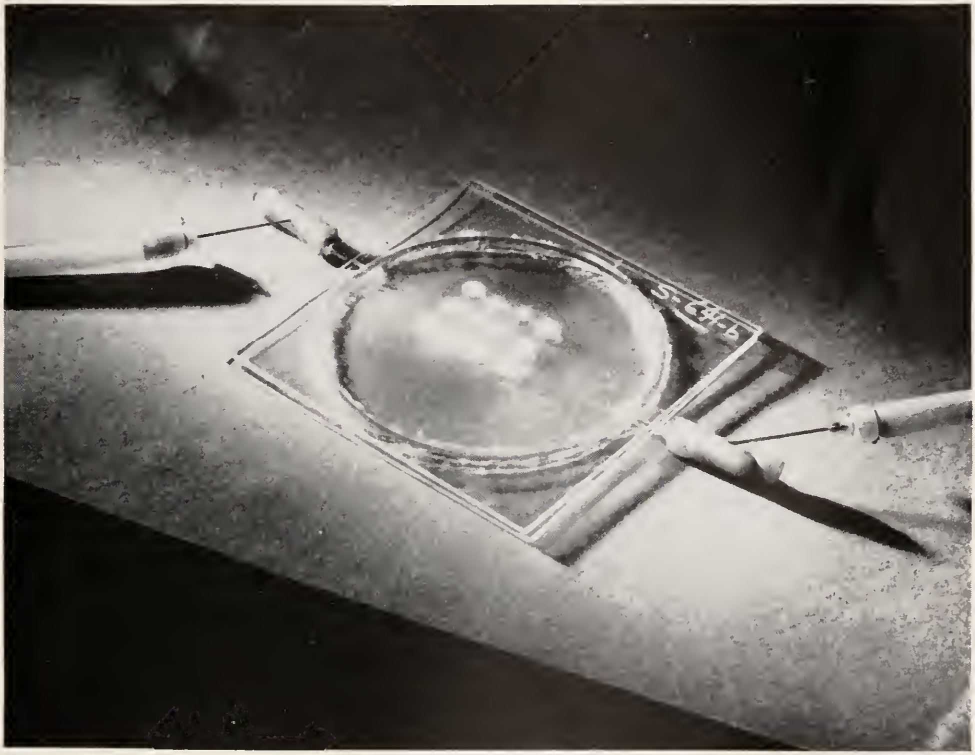

FIGURE 1. Culture chamber for studying the effects of various gases on sitting-drop preparations.

2. *Quantitative determination of the rate of spermatogenesis in a population of cysts* [3]

When suspended in blood containing the growth and differentiation hormone ("active blood"), the spermatocytes undergo meiosis and spermiogenesis during the course of about two weeks. Most of the cysts contain fully differentiated spermatozoa by the end of this period. In order to quantitate the rate of spermatogenesis, the stage of development of each cyst in the hanging- or sitting-drops was recorded at frequent intervals during the period of culture.

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[3] We gratefully acknowledge the assistance of Mr. Ned Feder in the development of this quantitative method.

Four successive stages in the development are easily recognized (see Fig. 1, Schmidt and Williams, 1953). Stage I includes primary and secondary *spermatocytal* cysts. Stage II includes spherical *spermatidal* cysts that have not yet begun to elongate. Stage III includes partially elongated *spermatidal* cysts less than twice as long as wide. Stage IV includes elongated *spermatidal* cysts more than twice as long as wide.

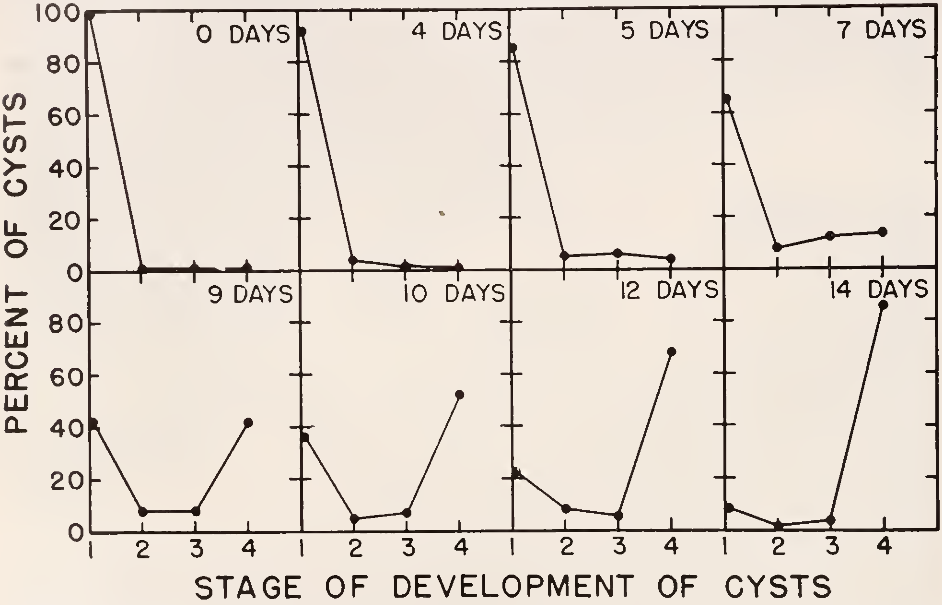

FIGURE 2. Day-to-day profiles during the normal development of a population of 600 cysts. For explanation see text.

As soon as a culture was prepared, the cysts were counted and identified under the low power objective of the compound microscope. This procedure was repeated at one- to three-day intervals. The results, when plotted, produced a series of "profiles" which described the composition of a particular population of cysts as a function of time.

The procedure is illustrated in Figure 2, which records the day-to-day profiles during the normal development of a population of approximately 600 cysts distributed in three hanging-drops. At zero days there was no development. But after five days, 19 per cent of the cysts had undergone meiosis and, of these, about 5 per cent had progressed to stage IV. Thereafter, the percentage of cysts in stage I continued to drop rapidly while that in stage IV increased. After nine days, the two stages were present in nearly equal numbers; after fourteen days, 86 per cent of the cysts had progressed to stage IV.

The determination of the relative rates of spermatogenesis (including both meiosis and spermiogenesis) under various experimental conditions was ordinarily carried out when 30 to 40 per cent of the cysts in a parallel control population had reached stages III or IV. The per cent of cysts in stages III or IV in the experimental population was compared with the corresponding determination on the control population. The relative rate of spermatogenesis was calculated as per cent of that observed in the control.

3. *Reagents*

Stock solutions were prepared in distilled water and the pH adjusted to 6.8–7.2 by the addition of NaOH or HCl. All heat-stable reagents were autoclaved before use. Heat-labile reagents were prepared in sterile distilled water and neutralized with sterile solutions of acid or base.

4. *Gases*

Compressed gases were obtained in commercial cylinders and assayed as follows:

Nitrogen (Airco), 99.5% nitrogen plus less than 0.5% oxygen. Ni~~trogen~~ (Linde), 99.99% nitrogen plus less than 0.01% oxygen. Oxygen (Airco), 99.5% oxygen plus less than 0.5% nitrogen. Carbon monoxide (Matheson Co.), 96.8% carbon monoxide, 0.36% carbon dioxide, 0.97% hydrogen, 1% nitrogen, 0.8% saturated hydrocarbons, 1.19 mg. iron/liter, 0.32 mg. sulfur/liter. Prior to its use, the carbon monoxide was filtered through 10% sodium hydroxide solution to remove the carbon dioxide and the iron carbonyl compounds.

Mixtures of oxygen, nitrogen, and carbon monoxide were prepared by compression in steel cylinders.

5. *Use of inhibitors*

A fresh suspension of cysts was prepared in active blood and divided into 0.09-ml. aliquots in small test tubes. To each tube was then added 0.01 ml. of an appropriate concentration of an inhibitor. The resulting 0.1-ml. mixture was drawn into a pipette and distributed in five drops of about 20 mm.^{3} each on a sterile 5″ × 5″ glass plate, as described in section 1. The preparations were then maintained at 25° C. in an incubator, and profile counts performed at intervals of one to three days. Three drops were selected containing a total of 300 to 600 cysts. The stage of development of each cyst was scored. Cysts and blood from the same series of donors were used in the preparation of parallel control cultures to which nothing save phenylthiourea was added. In the experimental results, the concentrations of the various inhibitors are recorded as the initial concentrations in the cultures. No attempt was made to define their distribution; *i.e.*, binding to cells, volatilization, or possible loss through degradation.

6. *Addition of gases*

About ten drops of a fresh cyst suspension were enclosed in the chamber illustrated in Figure 1. The glass plates were sealed with petrolatum and held together with clamps (not shown in the figure). Fifty to seventy-five volumes of an appropriate gas mixture saturated with water vapor were flushed through the chamber; the latter was then submerged in a water bath at 25° C. In experiments utilizing extremely low oxygen tensions, the chambers were flushed out daily to prevent the respiration of the cells from diminishing the oxygen tension significantly.

7. *Photo-reversibility of carbon monoxide inhibition*

In studies of the photo-reversibility of the carbon monoxide inhibition of spermatogenesis, a 150-watt incandescent lamp was utilized. The light was gathered by a reflector and focussed by means of a biconvex lens; it was then passed through a No. 3962 Corning filter and several centimeters of water to cut off the infrared. Each chamber was submerged in a 25° C. water bath and illuminated from above by a separate lamp. Control chambers were maintained in darkness by an envelope of aluminum foil.

RESULTS

Preliminary experiments were performed with each agent to define the range of concentration which inhibited spermatogenesis. Concentrations greater than 10^{-2} *M* were not investigated unless a specific activity at higher concentrations had previously been reported. A total of approximately 200 experiments was performed; each concentration of each agent was tested in duplicate or triplicate.

TABLE I

The effects of temperature on in vitro spermatogenesis

Temperature	Time required for initiation of development
10° C.	No development after 31 days
15° C.	23 days
20° C.	7 days
25° C.	3½ days
30° C.	3½ days

1. *Effects of temperature*

The results of the temperature studies are presented in Table I. When cysts were cultured at 10° C., no development was evident after 31 days. At 20° C. the first signs of development were detectable after 7 days, while at 25° C. and 30° C., development began in 3½ days. It is also of interest that after culture at 10° C. for as long as 15 days, the cysts resumed the normal tempo of development when returned to 25° C.

2. *Effects of metabolic inhibitors*

Figure 3 illustrates the results of a typical experiment in which suspensions of cysts were exposed for seven days to graded concentrations of an inhibitor—in this particular case, 2, 4-dinitrophenol. Development in the presence of 10^{-7} *M* proceeded at the same rate as that of controls; in 10^{-6} *M* there was definite inhibition; in 10^{-5} *M* only a few cysts underwent differentiation; in 10^{-4} *M* the cells were killed.

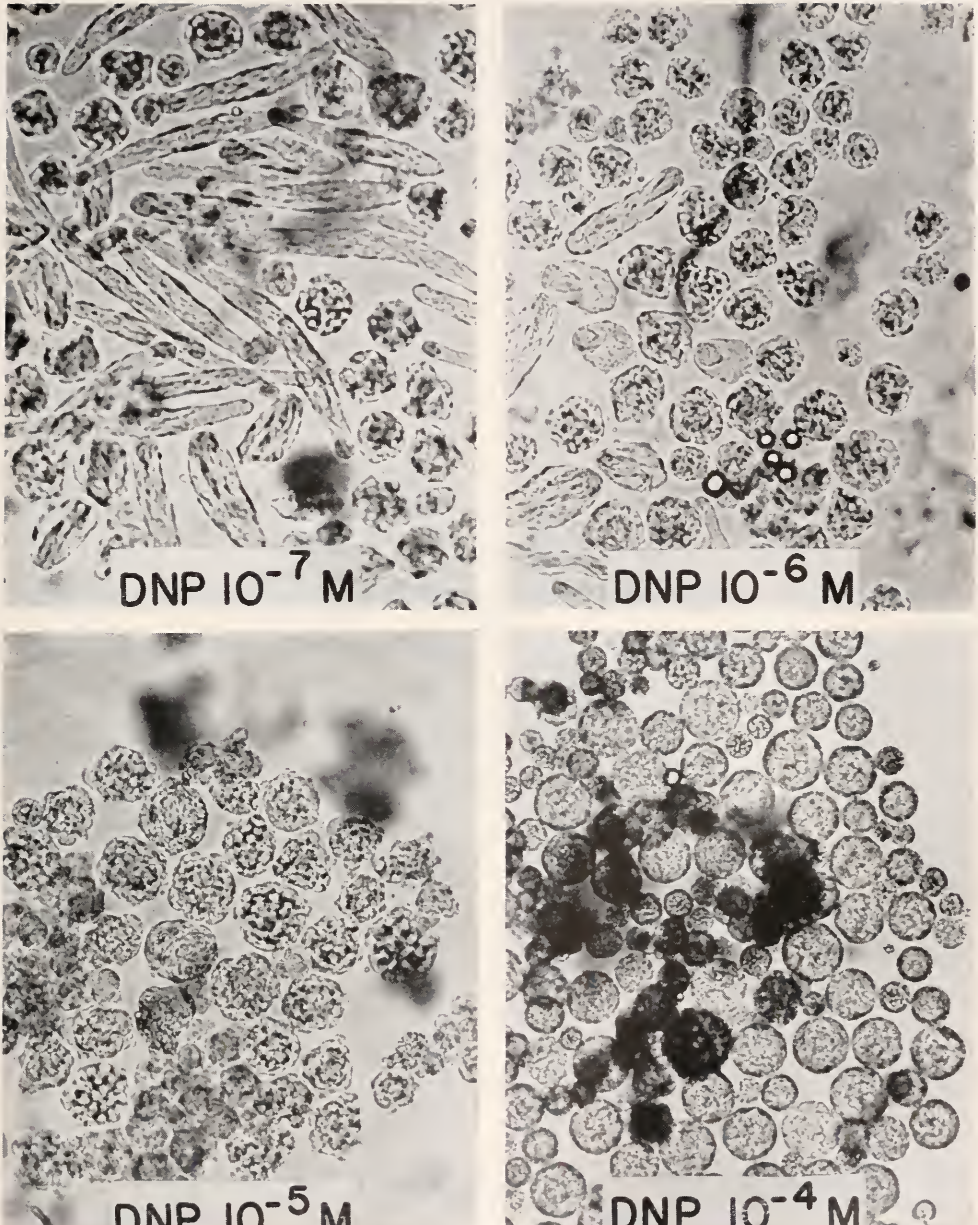
DNP 10^{-7} M
DNP 10^{-6} M
DNP 10^{-5} M
DNP 10^{-4} M

FIGURE 3. Fresh unstained cysts after seven days of culture in graded concentrations of 2, 4-dinitrophenol. × 65.

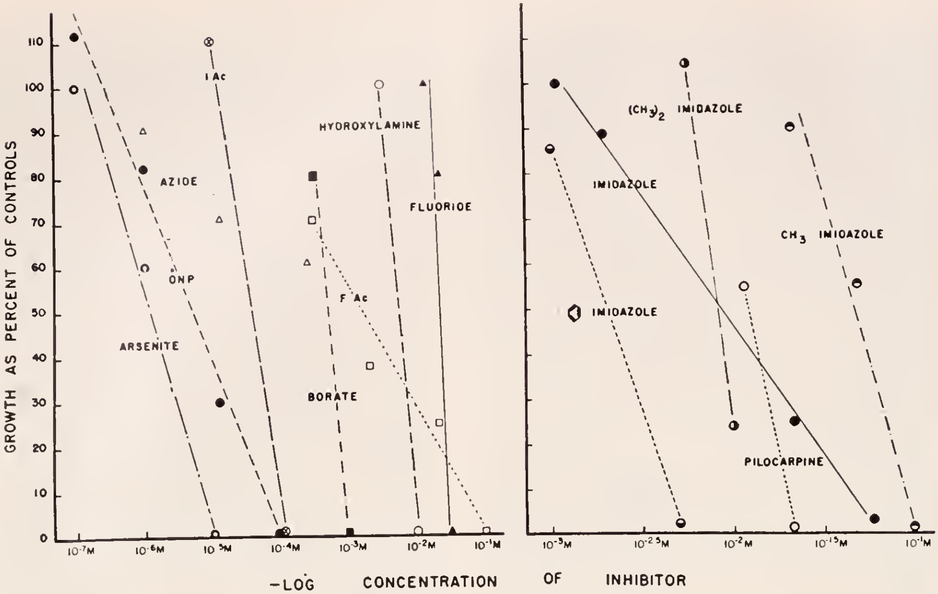

FIGURE 4. Effects of metabolic inhibitors on *in vitro* spermatogenesis.

TABLE II

The effects of twenty metabolic inhibitors on in vitro spermatogenesis (pH 6.8 to 7.2)

Inhibitor		Approximate concentration required for 50% inhibition of spermatogenesis ($\times 10^{-5}$ *M*)
1. Sodium arsenite		0.2
2. 2,4-dinitrophenol		0.7
3. Sodium iodoacetate		4
4. Sodium tetraborate		30
5. Sodium azide		80
6. Sodium fluoroacetate		80
7. Benzimidazole		200
8. Hydroxylamine hydrochloride		300
9. Semicarbazide hydrochloride		500
10. Imidazole		700
11. Dimethyl imidazole		700
12. Pilocarpine		1000
13. Sodium fluoride		3000
14. Methyl imidazole		5000
15. Ferrideuteroporphyrin		0.02 mg./ml.
16. Sodium pyrophosphate	No inhibition at	100
17. Coumarin	Slight inhibition at	500
18. Phenylthiourea*	No inhibition at	1000
19. Sodium malonate and malonamide	No inhibition at	5000

* All cultures were saturated with this drug for the purpose of blocking tyrosinase.

The quantitative results of a large number of similar experiments with different inhibitors are summarized in Figure 4. Development is recorded as per cent of that observed in control cultures as a function of the logarithm of the concentration of inhibitor. Although the range of effective concentrations differed considerably in the case of the several inhibitors, for each agent there appeared to be a roughly linear decrease in the rate of spermatogenesis as a function of the logarithm of inhibitor concentration. Table II tabulates the approximate concentrations of 20 chemicals that inhibited the rate of spermatogenesis by 50 per cent.

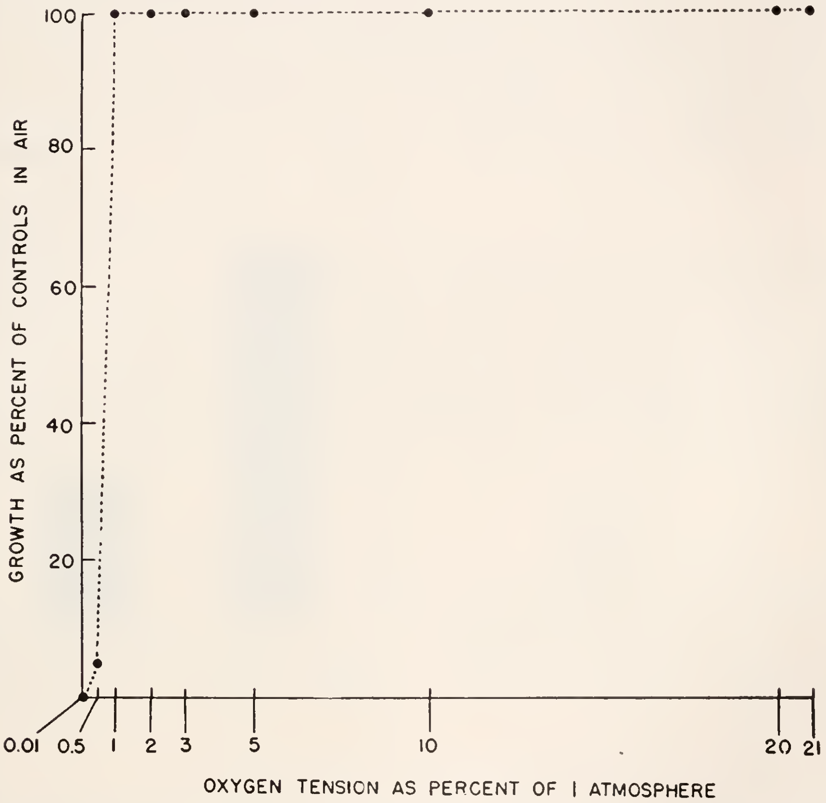

FIGURE 5. Diagrammatic summary of the effects of oxygen tension on *in vitro* spermatogenesis.

3. *Effects of oxygen tension*

Experiments testing the effects of oxygen tension on *in vitro* spermatogenesis are diagrammatically summarized in Figure 5. Oxygen at tensions between 21 per cent of an atmosphere (the normal tension in air) and 1 per cent of an atmosphere permitted spermatogenesis to proceed at the normal rate. As the oxygen tension was decreased below 1 per cent, considerable inhibition was observed. In 0.5 per cent oxygen the rate of development was only about 5 per cent of normal; in 0.01 per cent oxygen, development was completely inhibited.

4. *Effects of carbon monoxide*

Figure 6 records the effects of carbon monoxide on spermatogenesis. It will be observed that the inhibition depended not only on the pressure of carbon monoxide, but also on the pressure of oxygen that was simultaneously present. Thus, while a mixture of 90 per cent carbon monoxide plus 10 per cent oxygen (9:1) retarded the rate of development by 70 per cent, 90 per cent carbon monoxide plus 5 per cent oxygen (18:1) caused a 98 per cent inhibition.

In another series of experiments, cysts were exposed to 90 per cent carbon monoxide plus 10 per cent oxygen for five days and then returned to air. In many cysts the inhibition was immediately reversed.

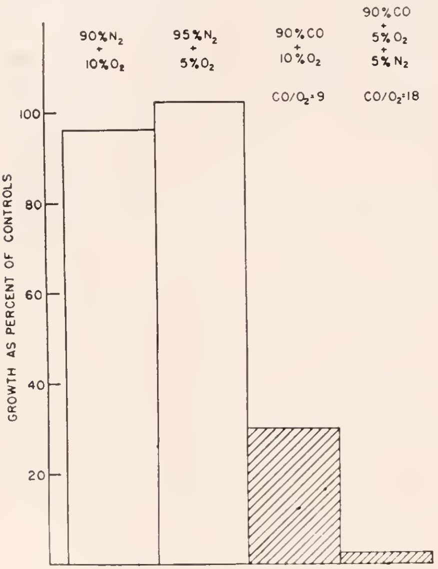

FIG. 6. Effects of exposure to various carbon monoxide/oxygen ratios on *in vitro* spermatogenesis in the dark.

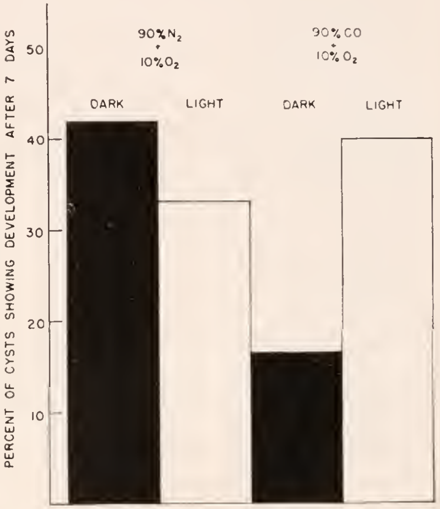

FIG. 7. Effects of light on the carbon monoxide inhibition of *in vitro* spermatogenesis.

5. *Photo-reversibility of the effects of carbon monoxide*

Because of their transparency the cysts afforded optimal material for testing the light reversibility of the carbon monoxide inhibition. Four chambers were used containing, respectively, 90 per cent nitrogen in the light, 90 per cent nitrogen in the dark, 90 per cent carbon monoxide in the light, and 90 per cent carbon monoxide in the dark. Ten per cent oxygen was present in all chambers. The results, recorded in Figure 7, show that light completely reversed the inhibitory effects of carbon monoxide.

DISCUSSION

The experimental results are relevant to considerations of the intermediary metabolism of both meiosis and spermiogenesis and to previous findings on intact insects.

1. *Temperature*

In vitro spermatogenesis showed substantially the same response to temperature as was encountered in the adult development of the animal as a whole. Both proceed most rapidly at environmental temperatures of 25° to 30° C. (Williams, unpublished observations) and are reversibly inhibited by low temperatures.

2. *Glycolytic system*

Though the anti-glycolytic agents, fluoride and iodoacetate, are not wholly specific, their potent inhibition of spermatogenesis suggests that the usual hexose-triose pathway of carbohydrate metabolism operates in the sex cells. However, it is clear that spermatogenesis is an aerobic process; the glycolytic reactions are therefore presumably concerned with the mobilization of substrates for oxidative breakdown.

3. *Tricarboxylic acid cycle*

The inhibition by arsenite, fluoroacetate, hydroxylamine, and iodoacetate is good evidence that the tricarboxylic acid cycle is utilized in spermatogenesis. In intact Cecropia pupae and developing adults, several components in this cycle have previously been demonstrated; namely, succinic dehydrogenase, malic dehydrogenase, and fumarase (unpublished observations) and coenzyme I (Jencks and Williams, unpublished observations). Moreover, fluoroacetate is known to be extremely poisonous for intact Cecropia at all stages in the life history (Williams, unpublished observations).

4. *Phosphate bond energy*

The inhibition by low concentrations of 2,4-dinitrophenol strongly suggests that spermatogenesis is energized by phosphate bond energy. This conclusion is consistent with the high toxicity of 2,4-dinitrophenol for intact Cecropia at all stages of development (Williams, unpublished observations).

We likewise suspect that the toxicity of azide is attributable, at least in part, to its inhibition of phosphate fixation (Loomis and Lipmann, 1949). Though azide is also known to inhibit the cytochrome system, the drug is extremely toxic when injected into diapausing Cecropia pupae (Williams, unpublished observations), where the function of the cytochrome system is not prerequisite to survival (Schneiderman and Williams, 1952).

5. *Terminal oxidases*

a. The experiments testing the effects of oxygen tension demonstrate that spermatogenesis is an aerobic process which apparently cannot be energized by glycolytic reactions. The short distances for the diffusion of oxygen in the *in vitro* preparations enabled the gas to saturate the terminal oxidase when the ambient gas phase contained oxygen at a tension of only 8 mm. of Hg. This finding contrasts sharply with that observed on the intact insect where oxygen is required to diffuse considerable distances through the tracheal system. Consequently, the adult development of Cecropia is inhibited when the ambient oxygen tension falls below 38 mm. Hg (5 per cent of an atmosphere) (unpublished observations). The fact that spermatogenesis proceeds at finite, though extremely low, tensions of oxygen is presumptive evidence that the terminal oxidase is cytochrome oxidase—an enzyme which, unlike other oxidases, is saturated at oxygen tensions between 0.25 and 2.5 mm. Hg (Winzler, 1941).

b. The failure of phenylthiourea, a powerful inhibitor of tyrosinase, to block spermatogenesis is in agreement with observations on intact Cecropia. Here phenylthiourea inhibits neither adult development (Williams, unpublished observations) nor respiration (Sussman, 1952). It seems safe to conclude that tyrosinase is not a terminal oxidase in processes responsible for the development of Cecropia *in vitro* or *in vivo*.

c. According to Pappenheimer and Williams (1952), certain imidazoles such as pilocarpine exert their effects by complexing ferriprotophyrin, thereby making this prosthetic group unavailable for the synthesis of heme-containing enzymes such as the cytochromes. The inhibitory action of these imidazoles on spermatogenesis (Fig. 4) roughly parallels their effects in blocking the development of the intact insect.

d. The inhibition of spermatogenesis by ferrideuteroporphyrin is probably attributable to its competitive inhibition of ferriprotoporphyrin (Pappenheimer and Williams, 1952). This inference once again points to the role of enzymes containing ferriprotoporphyrin in the metabolism of spermatogenesis; *e.g.*, certain components of the cytochrome system such as cytochrome *b*. The effects observed *in vitro* are analogous to those observed in intact Cecropia where ferrideuteroporphyrin inhibits adult development and other processes requiring cytochrome-mediated respiration.

e. Carbon monoxide's inhibition of spermatogenesis directly parallels its action on intact Cecropia during the period of adult development (Schneiderman and Williams, 1952). Since *in vitro* spermatogenesis was resumed upon return to air, it is clear that carbon monoxide blocked or retarded development without killing. The carbon monoxide inhibition of spermatogenesis was dependent on the carbon monoxide/oxygen ratio. The same finding has been demonstrated for the inhibition of adult development in the intact insect. Finally, the carbon monoxide inhibition of *in vitro* spermatogenesis was reversed by light—an effect which we have duplicated in the intact insect and shall describe in a subsequent communication.

Only one substance is affected by carbon monoxide in this manner; namely, cytochrome oxidase. We therefore conclude that cytochrome oxidase is the terminal oxidase in the morphogenesis of the testicular tissue, as in the insect as a whole.

The photograph in Figure 1 was made in collaboration with Dr. Roman Vishniac and is used with the permission of *Time*, Inc.

SUMMARY

1. The effects of temperature, metabolic inhibitors, oxygen tension, and carbon monoxide were studied in relation to the *in vitro* spermatogenesis (meiosis and spermiogenesis) of the male sex cells of the Cecropia silkworm.

2. A method is described for the quantitative study of the rate of spermatogenesis.

3. The optimum temperature for spermatogenesis is 25° to 30° C.

4. An appraisal of the effects of twenty diverse metabolic inhibitors revealed that the glycolytic system mobilizes substrates for spermatogenesis, that the tricarboxylic acid cycle is apparently the main pathway of acetate utilization, and that oxidative phosphorylation energizes spermatogenesis.

5. Spermatogenesis is shown to be an aerobic process, the terminal oxidase mediating the respiration being saturated by oxygen at tensions lower than 8 mm. of Hg.

6. Spermatogenesis is blocked or retarded by several inhibitors of the cytochrome system; the inhibition by carbon monoxide is light-reversible.

7. The findings on the isolated sex cells are compared to the effects of the same series of agents on the intact insect. The biochemical systems responsible for the meiosis and differentiation of spermatocytes *in vitro* are apparently the same as those which serve the development of the insect as a whole.

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CHOLINESTERASE AND LIPASE IN THE AMOEBOCYTES, INTESTINAL EPITHELIUM AND HEART MUSCLE OF THE QUAHOG, VENUS MERCENARIA[1]

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The isolated heart of the quahog, *Venus mercenaria,* has been shown by Prosser (1940) to be highly sensitive to acetylcholine and has been used by Welsh (1943) for the biological assay of acetylcholine. This preparation is particularly useful due to its great sensitivity to acetylcholine, resistance to changes in pH and rapid recovery. Thresholds for inhibition of the spontaneous beat of the isolated heart by acetylcholine are of the order of 10^{-10} to 10^{-11} gm./ml. Treatment of the heart with cholinesterase inhibitors (physostigmine, prostigmine or di-isopropyl fluorophosphate) results in a two to five times increase in inhibition of the strength of the heart beat when acetylcholine is added subsequently (Welsh and Taub, 1948). This slight potentiation is attributed to the low cholinesterase activity reported in the *Venus* heart by Jullien *et al.* (1938) and Smith and Glick (1939).

Augustinsson (1946a) showed that the tissues of the mollusc *Mya arenaria* hydrolyzed acetylcholine, benzoylcholine and acetyl β methyl choline and that the blood of the snail *Helix pomatia* hydrolyzes these substrates (1946b). Mendel, Mundell and Rudney (1943) reported that benzoylcholine is hydrolyzed by serum cholinesterase[3] but not by acetylcholinesterase or esterase and that acetyl-β-methyl choline is hydrolyzed only by acetylcholinesterase.

In a series of unpublished experiments concerned with the staining of the *Venus* heart with supravital dyes, questions arose regarding the localization of esterase activity in the heart, and the association of esterase activity with cell components which stained with Janus green B.

After staining whole *Venus* hearts in Janus green B 1 : 10,000 in sea water for two hours at 19° C., the ventricular and atrial muscle fibers were stained light blue-green. The cut end of the intestine was intensely stained, but little staining was observed in the circular muscle of the intestine. Scattered throughout the heart muscle, numerous amoebocytes were filled with dark blue-green granules. Reduction of the blue-green dye occurred when fragments of atrial muscle were spread under a cover slip on a microscope slide. The blue-green color of di-ethyl safranin dimethyl anilin (Janus green B) changed to the pink-red color of di-ethyl safranin,

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[1] This work was supported in part by a research grant from the Division of Research Grants and Fellowships of the National Institutes of Health, U. S. Public Health Service.

[2] Jeffries Wyman Scholar, Harvard University, 1952–53.

[3] Serum cholinesterase is synonymous with non-specific cholinesterase, pseudo-cholinesterase, type II cholinesterase, and s-type cholinesterase. Human serum, dog muscle, guinea pig liver, rat liver and numerous other tissues are capable of attacking benzoylcholine at various rates. Acetylcholinesterase is synonymous with specific cholinesterase, type I or true cholinesterase. Esterase is synonymous with non-specific esterase or aliesterase.

the reduction product. This indicates that hydrogen donor enzymes are probably present in the amoebocyte granules.

Structures which are known to contain esterase or cholinesterase and which stain with Janus green B occur in motor end-plates (Couteaux, 1947) and mitochondria (Zacks and Welsh, 1951). The appearance of intercostal muscle end-plates stained supravitally by Janus green B and for cholinesterase is similar. In both cases, the edges of the subneural apparatus of the end-plate are reactive. Rat liver mitochondria, which stain selectively with dilute solutions of Janus green B, also contain serum cholinesterase (Zacks and Welsh, 1951). The basis of this parallelism in staining may be due to the quaternary nitrogen groups of the basic dyes which mimic the "cationic head" of acetylcholine. Other evidence for the specific combination of Janus green B with cholinesterases and "receptor" substance of the *Venus* heart and frog end-plates occurs in the strong inhibitory actions of Janus green B on cholinesterase (Massart and Dufait, 1941) and the blocking activity of Janus green B and other basic dyes in the frog rectus abdominis preparation (unpublished observations).

The Janus green B staining granules of amoebocytes may well be regarded as mitochondria since they stain with this dye, contain hydrogen donor enzymes and stain with mitochondrial stains. Further investigations on the histochemistry of *Venus* amoebocytes will appear elsewhere.

The esterase and lipase activity of the amoebocytes and alimentary tract of several bivalve molluscs has been studied. Yonge (1926b) reported that amoebocytes from the oyster *Ostrea* were able to digest olive oil and fish erythrocytes, thus indicating the presence of intracellular lipase and protease. The ingestion and digestion of olive and peanut-oils were confirmed in the oyster, *Crassostrea virginica*, by George (1952). Takatsuki (1934) found difficulty in following the hydrolysis of olive oil in the amoebocytes of *Ostrea* but observed that methyl acetate was more readily hydrolysed. This finding probably indicates the presence of esterase. Yonge (1923) reported that lipolytic enzymes are absent in the midgut of the clam *Mya*. Details of the physiology and biochemistry of amoebocytes may be found in the work of Yonge (1923, 1926a, 1926b, 1929, 1946), Ohuye (1938), and the review of Haughton (1934).

In view of the early reports of lipase and esterase in amoebocytes and intestine of various molluscs and the selective staining of amoebocyte mitochondria by Janus green B, an investigation of the enzymes present in the isolated *Venus* heart, amoebocytes and intestine was of interest.

This investigation is divided into two parts. The first is concerned with evidence of enzymatic activity in the *Venus* heart and isolated amoebocytes obtained by manometric and biological methods. The second part presents evidence of enzymatic activity obtained through the use of recently developed histochemical methods.

PART I

METHODS

Five *Venus* hearts were excised with an enclosed length of intestine and blotted to remove surface moisture. The combined net weight was 728 mg. The hearts were then homogenized for 25 seconds in 30 ml. of sea water in a Waring Blendor.

A modified version of the standard (Ammon, 1934) manometric procedure for the determination of cholinesterase activity was used. Warburg vessels with side arms were filled with 3 ml. of a solution composed of 25 ml. sea water, 5 ml. 1.26% $NaHCO_3$ and 0.5 ml. of a solution of acetylcholine bromide 8 mg./ml. or benzoylcholine 8 mg./ml. The final acetylcholine concentration in each vessel was 1 mg./ml. Aliquots (0.5 ml.) of the whole heart homogenate were added to the side arm of the flasks. Another 0.5-ml. aliquot of homogenate was dried at 60° C. for 24 hours and the true dry weight was obtained by subtracting the weight of the salts in the aliquot. Four vessels were filled by the method described above and automatic correction of spontaneous hydrolysis was provided by employing a thermobarometer containing substrate but not homogenate. The vessels were equilibrated 10 minutes at 21° C., gassed with a mixture of 95% nitrogen and 5% carbon dioxide for three minutes and re-equilibrated for another 10 minutes. At the end of this period, the homogenate was tipped in and readings taken at 10-minute intervals for 60 minutes. Q_{Ach} values were calculated on the basis: microliters CO_2/mg. dry weight × hr.

RESULTS

Since the cholinesterase activity of the hearts is variable and the relatively low activity is on the threshold of accurate detection by this procedure, a sample experiment will be reported and the average results considered (Table I).

TABLE I

		Acetylcholine bromide 1 mg./ml.	Benzoylcholine 1 mg./ml.
Vessel	I	2.2	4.6
	II	9.0	7.9
	III	3.2	4.0
	IV	5.1	—
		Average: 4.8 (Q_{Ach} 0.61)	5.5 (Q_{Bzch} 0.78)

As can be seen from Table I, these average values ($Q_{Ach} = 0.61$; $Q_{Bzch} = 0.78$) are quite low compared with a Q_{Ach} of 34 for horse serum (Mendel and Rudney, 1943) or the average Q_{Ach} of 1.42 for isolated rat liver mitochondria (Zacks and Welsh, 1951). Also, the average values found in this study are lower than the Q_{Ach} of 5.5 reported by Smith and Glick (1939) calculated on the basis of microliters of CO_2/30 minute × mg. weight. According to the criteria of Mendel, Mundell and Rudney (1943), the splitting of benzoylcholine by *Venus* heart homogenates indicates the presence of serum cholinesterase. Acetylcholinesterase and esterase do not attack benzoylcholine according to these authors.

The confirmation of low serum cholinesterase activity in *Venus* heart homogenates was achieved, but the localization of the enzyme in the several tisuses included in the homogenate required further investigation. In practice, a length of intestine as well as numerous amoebocytes is included in the heart preparation used for the assay of acetylcholine and in the whole heart homogenates, so that attention must be directed to these component tissues.

In order to determine if the numerous amoebocytes in the heart contributed to the cholinesterase activity of the homogenates, isolated and washed amoebocytes were prepared and assayed for cholinesterase activity.

Five ml. of *Venus* blood were obtained by ventricular aspiration of several quahogs. The pooled blood was centrifuged at 700 × g. for 10 minutes to sediment the amoebocytes. The supernatant was decanted and stored at 4° C. in the refrigerator until it could be assayed. The sedimented amoebocytes were taken up in sea water and re-sedimented in order to wash away adhering serum. This procedure was repeated and the sediment was diluted with 5 ml. sea water. To 7 ml. of sea water, two ml. amoebocyte concentrate and one ml. 10^{-4} *M* acetylcholine bromide were added. This mixture was incubated at 27° C. for two hours and 0.1-ml. aliquots were tested on the *Venus* heart preparation (Welsh and Taub, 1948). Similarly, aliquots of mixtures containing separated serum were also tested. Control incubation mixtures were prepared which contained 6 ml. sea water, two ml. amoebocyte concentrate, one ml. physostigmine salicylate 10^{-3} *M* and one ml. acetylcholine bromide 10^{-4} *M*. After two hours incubation, mixtures were added to regularly beating *Venus* hearts in a bath containing 9 ml. sea water. The final concentration of acetylcholine was 10^{-5} *M* in the control mixtures. The concentration of acetylcholine added to the incubation mixture was chosen so that the final concentration in the heart bath, 10^{-7} *M*, if there had been no hydrolysis of acetylcholine, would produce complete inhibition of the heart beat. In a typical experiment, test concentrations of acetylcholine were added to the bath containing the beating heart to measure the sensitivity of the preparation. After washing and a 5-minute rest period, test aliquots of the incubation mixtures were added to the baths. After washing and rest, control concentrations of acetylcholine were added to the baths to test the recovery of the test hearts.

TABLE II

Summary of the composition of incubation mixtures used to detect cholinesterase activity

Number	(Ach) in mixture	Aliquot ml.	(Ach) in heart bath	Serum in mixture	Amoebocytes in mixture ml.	Physostigmine *M*	% inhibition
1	—	—	10^{-8} *M*	—	—	—	100
2	10^{-5} *M*	0.1	10^{-7}*	—	2	—	0
3	—	—	10^{-7}	—	—	—	100
4	10^{-5}	0.1	10^{-7}*	—	2	—	0
5	10^{-5}	0.1	10^{-7}*	—	2	10^{-4}	100
6	10^{-5}	0.1	10^{-7}*	—	2	—	0
7	10^{-5}	0.1	10^{-7}*	—	2	—	0
8	10^{-5}	0.1	10^{-7}*	2	—	—	0
9	10^{-5}	1.0	10^{-6}*	2	—	—	14
10	—	—	10^{-8}	—	—	—	100

* If there had been no hydrolysis of acetylcholine.

Table II summarizes the manner in which the various incubation mixtures in a sample experiment were prepared. The effect of the various mixtures on the test hearts can be seen by comparing the number of the mixture in Table II with the corresponding number in Figure 1.

Figure 1 is a typical record of a sample experiment. It can be seen that the amoebocyte concentrate and the supernatant serum produced nearly complete hydrolysis of the acetylocholine present in the incubation mixtures in two hours as evidenced by the failure of test aliquots to inhibit the spontaneous beat of the isolated heart. The same final concentration of acetylcholine (10^{-7} M) added to the bath and aliquots of incubation mixtures protected with 10^{-4} M physostigmine salicylate, completely inhibited the beat of the *Venus* hearts tested. Test concentrations of acetylcholine (10^{-7} M), added to the test hearts after the experimental aliquots had been tested, showed that the test hearts were still sensitive to acetylcholine.

Thus, isolated and washed *Venus* amoebocytes as well as serum contain enzymes capable of hydrolyzing acetylcholine. The observed complete inhibition of these enzymes by physostigmine salicylate 10^{-4} M indicates the presence of cholinesterase, since lipase and esterase are slightly inhibited by this concentration of physostigmine (Easson and Stedman, 1937; Richter and Croft, 1942). The presence of lipase or esterase in *Venus* amoebocytes cannot be excluded without additional evidence. The histochemical data, which will be presented below, indicate that a mixture of enzymes exists in the amoebocytes as well as in the intestine and heart muscle.

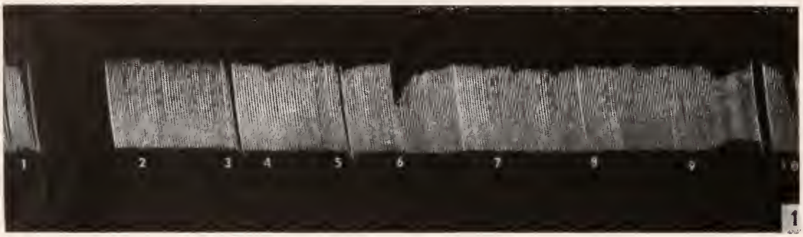


FIGURE 1. Sample record of the response of the isolated Venus heart to aliquots of incubation mixtures, to detect the presence of acetylcholine.

PART II

In order to obtain more information concerning the intracellular localization of esterases, recently developed histochemical methods were applied to amoebocytes, intestinal epithelium and heart muscle of *Venus*. These procedures have been used in combination with various enzyme inhibitors in an attempt to characterize the types of enzymes present.

METHODS

Nachlas and Seligman (1949) developed a procedure for the histochemical localization of esterase and lipase in acetone-fixed tissues. This method is based on the hydrolysis of β-naphthyl acetate by esterase and lipase. When β-naphthyl acetate is hydrolyzed, the resulting product is spontaneously converted to β-naphthol which, in the presence of a stabilized diazotised salt, precipitates as a granular, insoluble pigment at or near the site of enzymatic activity. Later, it was found that β-naphthyl acetate also was attacked by the enzymes of fresh, unfixed nervous tissue and that the failure of nervous tissue to react in the original method was due to the destruction of acetylcholinesterase by acetone. The modified procedure (Ravin, Zacks and

Seligman, 1953) utilizes the 6-brom derivative of β-naphthyl acetate as the substrate and tetrazotized anisidine (diazo blue B) as the coupling agent in the staining of fresh frozen sections cut by the Linderstrøm-Lang method as modified by Coons *et al.* (1951). Sections cut at 10 micra can be stored at 4° C. for one or two weeks without marked decline in enzymatic activity. When applied to fresh, frozen sections, this method will demonstrate lipase, esterase and cholinesterases. The particular enzyme present is determined by pre-treatment of the sections for one hour in various inhibitors known to differentiate lipase, esterase and cholinesterases.

Sodium taurocholate (10^{-2} *M*, 10^{-3} *M*) was used to differentiate lipase from esterase since, according to Willstatter and Memmen (1924) and Nachlas and Seligman (1949), esterase is inhibited, but lipase is activated or unaffected by this reagent. Advantage was taken of the observations that acetylcholinesterase and serum cholinesterase are inhibited by low concentrations of physostigmine (10^{-5} *M*) while esterase is little affected by concentrations as great as 10^{-3} *M* (Easson and Stedman, 1937; Richter and Croft, 1942). Also cold acetone (4° C.) applied for 24 hours destroys cholinesterase activity but lipase and esterase activity are relatively unaffected (Nachlas and Seligman, 1949). Other inhibitors which are claimed to differentiate between the various enzymes considered were also used.

Quinine (5×10^{-2} *M*) was used by Nachlas and Seligman (1949) who found that pancreatic hydrolysis of β-naphthyl acetate was moderately inhibited as was esterase activity in liver and kidney. These authors conclude that quinine, in this concentration, inhibits lipase more than esterase. Also, Augustinsson (1948) reported that quinine hydrochloride (3.78×10^{-4} *M*) strongly inhibited the hydrolysis of choline esters and tributyrin catalyzed by serum and liver preparations. Due to this lack of clearly specific inhibition produced by quinine, this reagent is less valuable for the characterization of the enzymes present in the *Venus* heart. Quinine alkaloid was used in a concentration of 10^{-3} *M*.

Arsenilic acid (atoxyl) 10^{-1} *M* was used by Nachlas and Seligman (1949) to inhibit esterase in homogenates of rat kidney, liver and pancreas and other tissues. The high concentration required attests to the low specificity of this reagent. Thus, to differentiate the main recognized groups of enzymes (lipase, esterase and cholinesterase), reliance was placed on the use of sodium taurocholate, physostigmine and cold acetone as the most reliable criteria available.

As a check on the accuracy of localization of enzymatic activity demonstrated by the use of 6-brom-2-naphthyl acetate, the method of Barrnett and Seligman (1951) was also used. In this procedure, the substrate employed is indoxyl acetate, which, when hydrolyzed by lipase, esterase or cholinesterase, yields indoxyl which oxidizes in air to form an insoluble precipitate of indigo at or near the site of enzymatic activity. The enzymes which attack this substrate are the same as those which hydrolyze 6-brom-2-naphthyl acetate and therefore must also be controlled by the use of inhibitors.

To test for the presence of serum cholinesterase, the method of Ravin, Tsou and Seligman (1951) was used. This procedure employs carbonaphthoxycholine iodide as the artificial chromogenic substrate. The coupling agent (diazo blue B) is the same as that used with 6-brom-2-naphthyl acetate. Carbonaphthoxycholine is hydrolysed by serum cholinesterase, but not by esterase, lipase or acetylcholinesterase.

The histochemical methods described above were applied to fresh whole hearts from which thin spreads were made of atrial muscle or to frozen sections, cut at 10 micra in the Linderstrøm-Lang cryostat. The sections were incubated in a Michaelis barbital buffer (pH 7.4 for 6-brom-2-naphthyl acetate and carbonaphthoxycholine and pH 8.4 for indoxyl acetate) containing the substrate, diazo blue B, NaCl and $CaCl_2$ as recommended in the respective methods. In inhibition experiments, the inhibitors were made up in the proper buffer mixture and slides were incubated in the solutions for 60 minutes. At the end of this time, the inhibitor solution was poured off into a flask and substrate and coupling agent were added. The mixture was then filtered back into the coplin jar containing the sections. In all cases, the reaction was carried out in the presence of the inhibitor to avoid reversibility of inhibition due to washing out of the inhibitor. The reaction was allowed to proceed for 20 minutes and the slides were washed in tap water and mounted in Kaiser's glycerogel. The slides were examined as soon as possible after staining since deterioration of the preparations occurred.

RESULTS AND DISCUSSION

Table III summarizes the results obtained in the histochemical examination of *Venus* amoebocytes, intestinal epithelium and heart muscle.

TABLE III

Hydrolysis of histochemical substrates by Venus amoebocytes, intestinal epithelium and heart muscle

Substrate	Amoebocytes	Intestinal epithelium	Heart muscle
6-brom β-naphthyl acetate	++	++	++
Indoxyl acetate	++	++	++
Carbonaphthoxycholine	++	+	++

After reaction with 6-brom-2-naphthyl acetate or indoxyl acetate, the amoebocytes were filled with blue or blue-violet punctate granules. Red-violet granules were seen in the amoebocytes after reaction with carbonaphthoxycholine iodide. The intestinal epithelium, especially the middle and basal regions of the tall columnar cells, was strongly reactive in many of the intestines examined as was the ventricular and atrial musculature. In other intestines, relatively little enzymatic activity was observed even though amoebocytes and heart muscle reacted normally. Apparently, these differences of enzyme activity in the intestinal epithelium reflect functional phases probably associated with digestion. Nuclei of amoebocytes, intestinal epithelium cells and heart muscle were never reactive.

Table IV summarizes the results obtained in the inhibition experiments. After pre-treatment with sodium taurocholate (10^{-3} M), no inhibition of amoebocytes or intestinal epithelium was observed although enzymatic activity in the ventricular muscle was partially inhibited. When a higher concentration of sodium taurocholate (10^{-2} M) was used, the intestinal epithelium and amoebocytes were still reactive, but the ventricular muscle enzymatic activity was completely inhibited. In a few experiments, it appeared that the reaction in the amoebocytes and intestinal epithelium after sodium taurocholate (10^{-3} *M*) had been increased and that activation of the enzymes present had occurred.

Physostigmine alkaloid (10^{-3} *M*) produced complete inhibition of all reactive tissues, but the same inhibitor tested in lower concentration (2×10^{-5} *M*) failed to inhibit the amoebocytes but did somewhat reduce the intensity of the reaction in the intestinal epithelium and more intensely inhibited enzymatic activity in the ventricular muscle. The results obtained with physostigmine (10^{-3} *M*) should be regarded with some doubt since the addition of diazo blue B to a physostigmine solution of this concentration results in the production of a deep orange solution which suggests that a reaction has taken place which may prevent diazo blue B from subsequently coupling with the substrate.

Acetone (4° C.) applied for 24 hours slightly inhibited the reaction in the intestinal epithelium and amoebocytes but markedly decreased the enzymatic activity of the ventricular muscle.

TABLE IV

Inhibition of Venus amoebocytes, intestinal epithelium and heart muscle by esterase and lipase and cholinesterase inhibitors

Inhibitor Substrate 6-brom β-NA	Amoebocytes	Intestinal epithelium	Heart muscle
Sodium taurocholate 10^{-3} *M*	++	+++	+
Sodium taurocholate 10^{-2} *M*	+	++	−
Quinine alkaloid 10^{-3} *M*	++	++	+
Physostigmine alkaloid 2×10^{-5} *M*	++	+	±
Physostigmine alkaloid 10^{-3} *M*	−	−	−
Atoxyl 10^{-1} *M*	−	−	−
Acetone 24 hr., 4° C.	+	++	±
Water 90° C., one min.	−	−	−

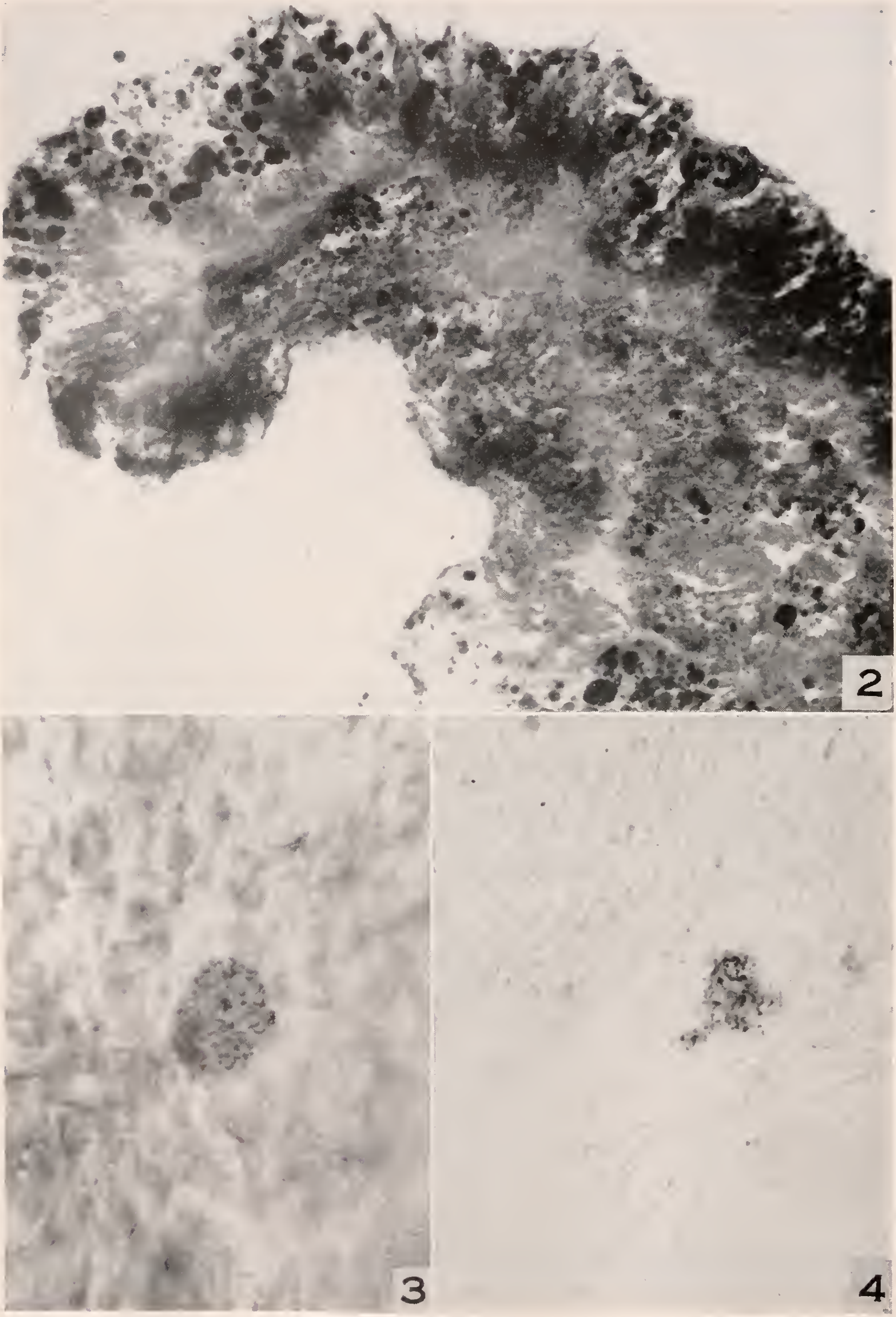
− Complete inhibition.
± Mostly inhibited, but few blue-violet granules present; considerably less than control.
+ Pink background, scattered blue-violet granules; less than control.
++ Fairly intense blue or blue-violet, punctate granulation; control level of staining.
+++ Very intense reaction; greater than control—activation.

Quinine alkaloid (10^{-3} *M*) failed to inhibit the amoebocytes or intestinal epithelium, but slightly decreased the reactivity of the ventricular muscle.

Arsenilate (10^{-1} *M*) completely inhibited all staining in the amoebocytes, intestinal epithelium and ventricular muscle.

When carbonaphthoxycholine iodide was used as the substrate, amoebocytes, intestinal epithelium and ventricular muscle were stained. The ventricular muscle was particularly reactive as indicated by the intense stain.

From the collected histochemical evidence summarized in Tables III and IV, it appears that lipase and serum cholinesterase are present in varying amounts in *Venus* amoebocytes, ventricular muscle and intestinal epithelium. The staining with carbonaphthoxycholine and the inhibition of staining with 6-brom-2-naphthyl acetate after pre-treatment with physostigmine (2×10^{-5} *M*), quinine (10^{-3} *M*) and cold acetone indicate that the activity of serum cholinesterase is greater in the ventricular muscle than in the amoebocytes or intestinal epithelium. Lipase activity is



Figures 2–4.

localized in the intestinal epithelium and in the amoebocytes and to a lesser extent in the ventricular muscle. This is shown by the failure of sodium taurocholate to inhibit amoebocyte and intestinal epithelium activity. Ventricular muscle was completely inhibited by 10^{-2} *M* sodium taurocholate. Lipase activity in the intestinal epithelium was not destroyed by cold acetone, while the ventricular muscle was completely inhibited.

The histochemical findings summarized above demonstrate the distribution of serum cholinesterase and lipase activity in the *Venus* heart and associated tissues, and account for the results recorded in the literature and in this study. The demonstration of lipase in amoebocytes by histochemical means confirms the observations of Yonge (1926b) who observed the digestion of oil and fish erythrocytes in *Ostrea*. The hydrolysis of acetylcholine and benzoylcholine by whole heart homogenates can be attributed to serum cholinesterase as demonstrated by carbonaphthoxycholine iodide. It has been shown by Ravin *et al.* (1951) that β naphthyl acetate is hydrolyzed by homogenates of rat brain, rat liver, washed human erythrocytes and by human serum and purified human serum cholinesterase but that carbonaphthoxycholine iodide is hydrolyzed only by human serum and purified serum cholinesterase. Ravin *et al.* (1951) point out the similarity of carbonaphthoxycholine and benzoylcholine as the basis for the hydrolysis of both substrates by serum cholinesterase. It is interesting that rat liver fails to attack carbonaphthoxycholine yet contains an enzyme capable of hydrolyzing benzoylcholine which is associated with the mitochondria (Zacks and Welsh, 1951). This may indicate that more than one type of serum cholinesterase exists and that the type found in *Venus* is more like the type found in human serum than the enzyme present in rat liver mitochondria. Takatsuki's observation (1934) that amoebocytes of *Ostrea* contain enzymes capable of hydrolysing methyl acetate may be interpreted in terms of serum cholinesterase activity or an esterase. The presence of esterase in the heart and intestinal epithelium of *Venus* can not be completely excluded on the basis of the histochemical experiments. However, the nearly complete protection of acetylcholine by physostigmine (10^{-4} *M*) in incubation mixtures containing isolated amoebocytes suggests that serum cholinesterase is the enzyme in the amoebocytes which attacks acetylcholine most readily.

The role of amoebocytes in intracellular digestion has been studied by Yonge (1923, 1926a, 1926b, 1946) who believes that the digestion of fat and proteins in molluscs is primarily intracellular in the cells of the digestive diverticula and in the

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FIGURES 2–4.

FIGURE 2. Fresh frozen transverse section through the intestine of *Venus mercenaria*, showing total enzymatic activity after 20 minutes incubation in a solution containing 10 mg. of 6-brom-2-naphthyl acetate and 40 mg. of diazo blue B in barbital buffer pH 7.4. Note the intense activity in the basal and midportions of intestinal epithelium. The dark globules of "excretory substance" do not contain enzymatic activity but are colored red by dissolved azo dye. Enzymatic activity in the epithelium is indicated by fine blue punctate granules of the precipitated dye. 100 ×.

FIGURE 3. View of a rounded-up amoebocyte, from a fresh spread of atrial muscle, containing granules of precipitated azo dye after 20 minutes staining with a mixture of β-naphthyl acetate and diazo blue B. 1000 ×.

FIGURE 4. An example of an amoebocyte with a characteristic pseudopod in a fresh spread of atrial muscle stained supravitally by β-naphthyl acetate and diazo blue B for 20 minutes at room temperature. 1000 ×.

amoebocytes. Thus, the amoebocytes are thought to have a major role in the absorption and digestion of fat and protein. Evidence of extracellular digestion of fats and proteins in the intestinal tract of several molluscs has been obtained by Sawano (1929), Mansour (1946) and Mansour-Bek (1946). George (1952), using Nile blue sulphate as an indicator, observed that oil droplets are hydrolyzed in the stomach of *Crassostrea virginica* and that neutral fat was hydrolyzed by minced crystalline style and digestive gland. This author was also able to confirm the ingestion and subsequent intracellular digestion of oil by the amoebocytes but concluded that the fat ingested was too little to be significant in nutrition.

The demonstration of variable levels of lipase and cholinesterase activity in the intestinal epithelium of *Venus* seems to lend support to the concept of extracellular digestion of fats in the intestinal tract of certain molluscs.

The role of serum cholinesterase, either in sera or tissues, is not known, although a digestive role might be suspected in *Venus* since the enzyme occurs in amoebocytes and intestinal epithelium.

SUMMARY

1. Whole *Venus* heart homogenates hydrolyze acetylcholine and benzoylcholine at a low rate.

2. The enzymatic activity of such homogenates is the sum of that contributed by amoebocytes, intestinal epithelium and heart muscle.

3. Isolated amoebocytes hydrolyze acetylcholine and the enzyme responsible is inhibited by 10^{-4} M physostigmine, thus indicating the presence of cholinesterase.

4. Recently developed histochemical procedures demonstrate serum cholinesterase and lipase in amoebocytes, intestinal epithelium and heart muscle.

5. Greatest lipase activity is present in the amoebocytes and intestinal epithelium, while serum cholinesterase activity is greatest in the ventricular muscle.

6. The potentiation of the action of acetylcholine on the isolated *Venus* heart by physostigmine is due to inhibition of serum cholinesterase in the amoebocytes and especially in the heart muscle.

7. The presence of varying levels of lipase in the intestinal epithelium of *Venus* lends support to the suggested presence of extracellular lipolytic enzymes in certain molluscs.

8. In *Venus* amoebocytes, another case is seen where cholinesterase activity is associated with structures which stain supravitally with Janus green B.

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FERTILIZATION AND ARTIFICIAL ACTIVATION IN THE EGG OF THE SURF-CLAM, SPISULA SOLIDISSIMA[1]

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In fertilization the sperm generally performs two functions: activation and syngamy; these functions are experimentally separable. Under conditions where artificial parthenogenesis appears to involve the same initial sequence of developmental events that follow fertilization, it is believed that the stimulating agent somehow imitates the activating action of the sperm. Consequently, the term *parthenogenesis* (natural or artificial) which implies cleavage and later development, has been included under the wider term *activation.* (For reviews on activation and fertilization, see Loeb, 1913; Dalcq, 1928a; Tyler, 1941; Lillie, 1941; Runnström, 1949; Brachet, 1950, Chapter IV; and Heilbrunn, 1952, Chapter XL.) In recent years egg activation has been considered also as a specific case in the general phenomenon of excitation. It is generally true that activating agents for eggs also are stimulating agents for other kinds of cells. For this reason it is logical to extend the same concepts of excitation to egg cells that have been applied to stimulation and response in nerve, muscle, or protozoan cells. Furthermore, work done on various phases of egg activation complements that being done on other kinds of cells.

A study of excitation requires that a response be detectable soon after application of the stimulating agent. The surf-clam egg has a great advantage in this respect, for it contains a large germinal vesicle, the membrane of which breaks down readily upon stimulation. Thus the breakdown of the nuclear membrane can be taken as a criterion of response. The presence of polar bodies or of pronuclei at about 45 minutes after activation was taken as evidence that the cell was alive.

Previous studies on artificial parthenogenesis, especially those involving the eggs of echinoderms, often employed cleavage as a criterion of activation. However, in *Spisula* eggs and other eggs where fertilization occurs before the onset of maturation division, activation is not always followed by cleavage.

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[1] Presented to the Faculty of the Graduate School of the University of Pennsylvania in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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The purpose of the present study has been to make a preliminary survey of fertilization and artificial activation in the surf-clam egg. Wherever possible, the approach has been from the standpoint of similarities and differences in the mechanisms involved in fertilization and artificial activation. Because *Spisula* eggs have been little used in previous studies of this kind, it was considered desirable to make observations on normal and parthenogenetic development, and also to repeat experiments performed by others on different eggs in order to establish a baseline for further work on the mechanism of activation.

Schechter (1941) described a method for obtaining gametes from surf-clams, and also the structural changes which occur in aging eggs. His paper also contains some observations on normal early development. The clam used by Schechter was *Spisula solidissima* (Dillwyn), although it has commonly been called *Mactra.* Kostanecki (1908) described artificial parthenogenesis in the eggs of *Mactra stultorum* and *M. helvacea;* these eggs were obtained from animals collected at Naples, Italy. The eggs of the species of *Mactra* studied by Kostanecki appear to be quite different in many respects from those of *Spisula solidissima.*

I wish to express my gratitude to Dr. L. V. Heilbrunn, under whose friendly guidance these studies were carried out, for helpful advice on experiments and assistance in the preparation of the manuscript. I wish also to thank Dr. H. G. Borei for his helpful criticisms of the manuscript. Thanks are also due to Dr. S. Inoué for the use of his polarization microscope and air-turbine centrifuge. This study was greatly aided through the kind cooperation of Mr. and Mrs. P. Breslin and Mr. F. Matthews of Belmar, New Jersey, in obtaining surf-clams and sea water.

MATERIAL AND METHODS

A. Material

Along the New Jersey coast, eggs of the surf-clam can readily be obtained at any time from early spring until late autumn. At Woods Hole, Massachusetts, the season is shorter, beginning late in May and extending into September. At Woods Hole there seems to be a period of one or two weeks in the middle of the season when gametes are scarce; this period often coincides with periods of excessive summer heat. The spawning of surf-clams can be induced during the winter months by warming them gradually and feeding them plankton over a period of about two weeks (Loosanoff and Davis, 1950).

For the experiments at the Zoological Laboratory, University of Pennsylvania, surf-clams were transported from a float in the channel of the Shark River Inlet in Belmar, New Jersey to Philadelphia, where they were maintained under aeration at about 14° C. in a constant temperature bath for one to three weeks. Four clams about four inches in length can be kept for a period of five to six days in one half gallon of sea water in a jar. It is particularly important to evacuate any air trapped in the gill chamber of the clams; otherwise death will result in a short time. This may be done by holding and gently squeezing each clam under water with its siphons facing upwards until all air bubbles have emerged. Natural sea water was employed in maintaining clams, and artificial sea water of the same salinity was used in all experiments with eggs in Philadelphia. In Woods Hole, filtered sea water or artificial sea water could be used for the experiments; clams were obtained from Barnstable and kept in tanks of running sea water.

1. *Determination of sex*

Dr. L. Thomas, in work done at this laboratory, found that by inserting a medium-gauge hypodermic needle between the valves, near the hinge into the visceral mass, gametes could be drawn out for microscopic examination. This procedure permits separation of the sexes if desired, saves time and material, and could also be used for obtaining small amounts of gametes.

2. *Obtaining gametes*

Large numbers of gametes are obtained by breaking the shell near the umbo, cutting the adductor muscles and removing the visceral mass intact. The gills, mantle, and heart are trimmed away to expose the gonad, which then is excised. A large portion of the gonad lies deep within the visceral mass toward the foot. An excised ovary is cut up in a small volume of sea water and strained through a pad of cheesecloth into a large beaker containing filtered sea water. The eggs are allowed to settle to the bottom and the supernatant fluid is siphoned off. This washing process is repeated at least three times (Allen, 1951a). Thorough washing removes most of the blood, cellular debris from the ovary, and fertilizin. The latter substance has an adverse effect on fertilization in this species (Allen, 1951c). Excised testes are stored in the refrigerator until needed. During this storage period (up to six hours) a milky fluid containing sperm flows out of the gonad. This sperm suspension ("dry sperm") may be drawn readily into calibrated pipettes, permitting an accurate measurement of sperm concentrations. Precise measurements of sperm concentrations are required for some aspects of work involving fertilization.

3. *Structure of the unfertilized egg*

The unfertilized *Spisula* egg has a diameter of 56 microns, measuring from inside the vitelline membrane. The jelly layer, which is about two microns thick, is thinner than that of most marine eggs. It can usually be observed only after the addition of a dye (such as toluidine blue or Janus green B) or as an invisible barrier separating crowded eggs. The tough, easily visible vitelline membrane surrounds a thin, clear layer which is optically empty under bright field illumination, but negatively birefringent in the radial axis of the egg. Beneath the thin, clear layer is a differentiated cortex containing large granules which fail to move when forces of 200,000 × gravity are applied with a Beams-type, air-turbine centrifuge. The cytoplasm is moderately granular and about 11% of its volume is occupied by fat-containing granules (centrifuge experiments). Centrifugation stratifies these fat granules centripetally in a dense layer long before the heavy granules begin to collect at the opposite end of the cell. The properties of the cytoplasm are such that movement of the nucleus by centrifugal force produces a ring of negative strain birefringence in the region of the cytoplasm through which the nucleus has passed (Allen, 1952a). The germinal vesicle is about 31 microns in diameter and is somewhat eccentric. In life the nuclear membrane exhibits weak negative radial birefringence; when fixed, it shows, distributed along its inner surface, the entire chromosome complement except that chromosome which is attached to the nucleolus. The chromosomes are minute tetrads. The nucleolus is double (amphinucleolus), consisting of a transparent nucleolus and an opaque nucleolinus (see Plate I, Fig. E.). These structures are approximately 11 and 3 microns in diameter, respectively. Similar double nucleoli have been seen in many other marine eggs in the oöcyte stage.

Freshly shed eggs are irregular in outline. However, by the time washing has been completed, they usually become spherical. Presumably this irregularity in shape is due to compression in the ovary.

4. *Fertilization and early development at 21° C.*

a. *Optimal conditions for fertilization.* As mentioned earlier (see also Allen, 1951a), it is important to wash Spisula eggs several times with filtered sea water before insemination. Failure to wash sufficiently (*i.e.,* to remove blood and fertilizin, etc.) will result in low percentages of fertilization and delayed or inhibited cleavage. Two other important considerations are egg and sperm densities. Stender dishes containing 10 ml. of sea water were placed in a constant temperature bath. One or two drops of washed concentrated eggs were placed in the dish, so that their number was approximately 200–300 per ml. Immediately, 0.05 ml. of a fresh 1 : 500 dilution of "dry sperm" in filtered sea water was added to each dish. The sperm density varies considerably and should be assayed in a counting chamber for accurate work. The final density should be corrected to about 10^5 cells/ml.

The time taken for penetration of the sperm into different eggs in a population can be inferred from the sigmoid curves obtained by plotting counts of germinal vesicle breakdown against time after insemination. Fifty per cent nuclear breakdown occurs at about 9½ minutes after insemination; the spread of the curve is about 2–2½ minutes. As the time lag between sperm penetration and nuclear breakdown is the same in every egg, this curve indicates that all of the eggs are probably fertilized within 2–2½ minutes after insemination, depending on the chance meetings of eggs and sperm in the suspension. Furthermore, increasing the sperm concentration increases the slope of the sigmoid curve, but high concentrations also tend to produce polyspermy. The slope of the curve often permits detection of lots of poor eggs or sperm.

b. *Normal development at 21° C.* Soon after fertilization some eggs exhibit a small indentation at one point on the surface. This is more pronounced in some lots of eggs than in others. At best it is seen in about a quarter of the eggs in a microscopic field, but as in ordinary microscopic observation only a portion of the egg surface can be observed at any time, it is likely that most or perhaps all of the eggs show this irregularity. Almost simultaneously, the vitelline membrane elevates gradually from the surface of the egg. This process differs from the membrane elevation of echinoderm eggs, for the cortical granules, which appear to be so important in the membrane elevation of the sea urchin egg, do not appear to participate in the elevation of the *Spisula* egg membrane. The cortical granules persist after fertilization and throughout early cleavages.

Six or seven minutes after insemination[3] the nucleus begins to change; the transparent nucleolus dissolves and releases its substances into the nucleoplasm. The nucleolinus comes to lie free in the nucleus, and remains in the vicinity of the forming maturation spindle even after the disappearance of the nuclear membrane. Observations on fixed material indicate a spindle-forming role for the nucleolinus (Allen, 1951b).

The first meiotic metaphase lasts from 13 to 26 minutes after insemination. During this time, the spindle migrates from the center to the periphery of the egg. The first polar body is extruded at 29 minutes and its position establishes the animal pole of the egg. At this time the animal pole flattens slightly, then rounds up again at 35 minutes. The second polar body appears 39 minutes after fertilization. There follows a second brief flattening before the male pronucleus becomes visible at 50 minutes. Within another minute the female pronucleus also appears, and the two nuclei swell almost simultaneously, to a diameter of 11–12 microns before they merge and break down, giving rise to a clear region in the center of the cell. At 69 minutes the eggs elongate parallel to the long axis of the cleavage spindle. Formation of the cleavage furrow begins at 71 minutes, and is complete by 74 minutes. The nucleus re-forms in the (smaller) AB cell at 77 minutes and in the (larger) CD cell at 82 minutes after insemination. At 89 minutes both nuclei disappear again almost simultaneously in preparation for the second division at 99 minutes. The second cleavage divides the A and B cells equally, but the C cell is smaller than the D cell. Further cell divisions are more rapid and somewhat difficult to observe in living material. Rotating blastulae are seen usually within four or five hours after insemination; swimming larvae within a day. Veliger larvae will live at least three weeks in artificial sea water without feeding. (For early stages of normal development, see Plates I and II.)

c. *Abnormalities.* Polyspermic eggs usually fail to cleave; sometimes, however, they divide into blastomeres of equal size, or develop into abnormal larvae. Presence of more than two pronuclei in fertilized eggs is an indication of polyspermy.

Occasionally the cytoplasm of fertilized eggs becomes dark in color after nuclear breakdown. The cause of this abnormality may be sea water impurities, unripe gametes or unhealthy clams. Lots of clams with eggs showing this dark cytolysis often exhibit high percentages of spontaneous germinal vesicle breakdown after about an hour.

Aging in *Spisula* eggs leads to two obvious changes: first, there often appear deep indentations on the surface of the eggs (Schechter, 1941); such eggs can usually be fertilized normally if not too old. Second, the nucleus loses some of its normal transparency. Although these changes are not significant with respect to development, they should be recognized as a factor possibly influencing the physiology of the egg (see Scott and Hayward, 1950).

An occasional lot of eggs shows clumping of the cytoplasmic granules. Such eggs were discarded although it was observed that they were often able to develop normally.

Under-ripe eggs often stick together in groups. This kind of agglutination means that the jelly has not completely formed and is sticky. Such eggs are not suitable for experimental work, even though they may be fertilizable.

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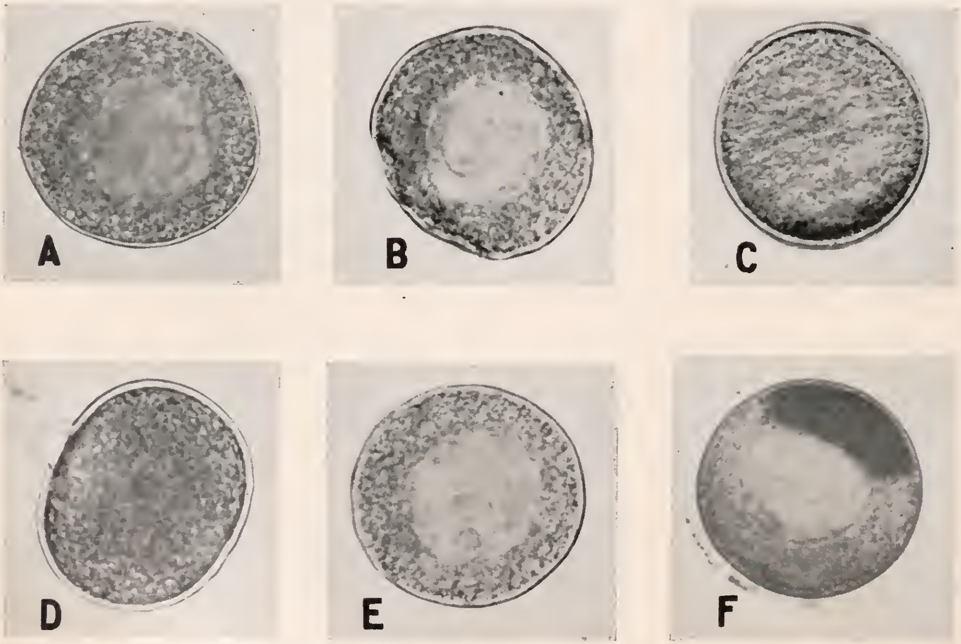
[3] All time figures given in this section refer to the time at which 50% of the eggs exhibit the particular phenomenon. This time is accurate to within a minute at constant temperature if egg batches from different females are compared under similar conditions. Under optimal conditions the spread of the points for eggs from a single female is about two minutes or less.

d. *Development of artificially activated eggs.* As far as could be determined, the early stages following artificial activation up to and including the breakdown of the nuclear membrane are identical with the early stages following fertilization. Furthermore, activated eggs usually form at least one polar body, often two; but with the methods tried so far, cleavage is rare. After one or two hours, activated eggs often exhibit from one to several nuclei, a condition highly suggestive of polyspermy. Such a multinucleate condition apparently comes about through a failure of the egg to form a second polar body; the second nucleus is retained in place of the male pronucleus. A similar situation was reported by Morris (1917) in the *Cumingia* egg, and by Kostanecki (1908) in *Mactra.* These nuclei sometimes undergo an abnormal mitotic reproduction without division of the cell; multipolar spindles are sometimes seen.

PLATE I. *Spisula solidissima* eggs



A. Unfertilized egg showing intact germinal vesicle.

B. Unfertilized egg shown 20 seconds after stimulation by isotonic sodium chloride in the presence of traces of calcium.

C. Egg 10 minutes after insemination showing the clear zone left after the dissolution of the germinal vesicle membrane.

D. Egg 30 minutes after insemination showing the first polar body; note the slightly elevated membrane (compare with B).

E. Unfertilized egg showing the double nucleolus in sharp focus.

F. A centrifuged egg showing the layer of light fat granules; note the sperm adhering to the surface of the jelly layer.

B. *Methods*

All experiments on artificial activation were carried out with widely dispersed eggs in order to eliminate crowding as a factor in excitation. A constant volume (10 ml.) of sea water or other medium was used throughout. Artificial activation experiments were carried out for the most part in petri dishes 5–6 cm. in diameter; water loss from evaporation was negligible. Experiments involving ether were carried out in stoppered vials. Since the threshold of stimulation depends on the temperature, this factor had to be controlled. For ordinary observations, room temperature (around 21 degrees) was sufficiently constant to eliminate errors from this source. For more exacting experiments a constant temperature water bath set at 21° (± 0.05°) was used.

The source of ultraviolet irradiation used in activation experiments was a Hanovia mercury arc lamp (Model number 7420) at a target distance of 35 cm. delivering approximately 4500 microwatts per square centimeter.

All solutions used in artificial activation experiments were isotonic to sea water with the exception of the mixtures used in osmotic stimulation. The exact isotonic concentration of sucrose used in centrifugation experiments could not be determined because the eggs always became very irregular in this medium. Unless stated otherwise, the pH of all solutions used was adjusted to 8.0–8.2 with 0.5 N NaOH. Mixtures of solutions and sea water are referred to as percentage (V/V) of isotonic solution in sea water (*e.g.*, "5% KCl-SW" means 5 volumes isotonic KCl and 95 volumes of sea water). In the figures, these percentage expressions have been converted to millimols of cation per liter of solution, since the concentrations of the other ions remain essentially constant.

Isotonic artificial sea water solutions were made up according to the formulas presented below, which were recalculated and modified after Lyman and Fleming (1940). Artificial sea water solutions lacking various of the cations were also used. For unbuffered solutions, bicarbonate was omitted. All solutions were prepared from water distilled in an all-Pyrex apparatus containing potassium permanganate and phosphoric acid.

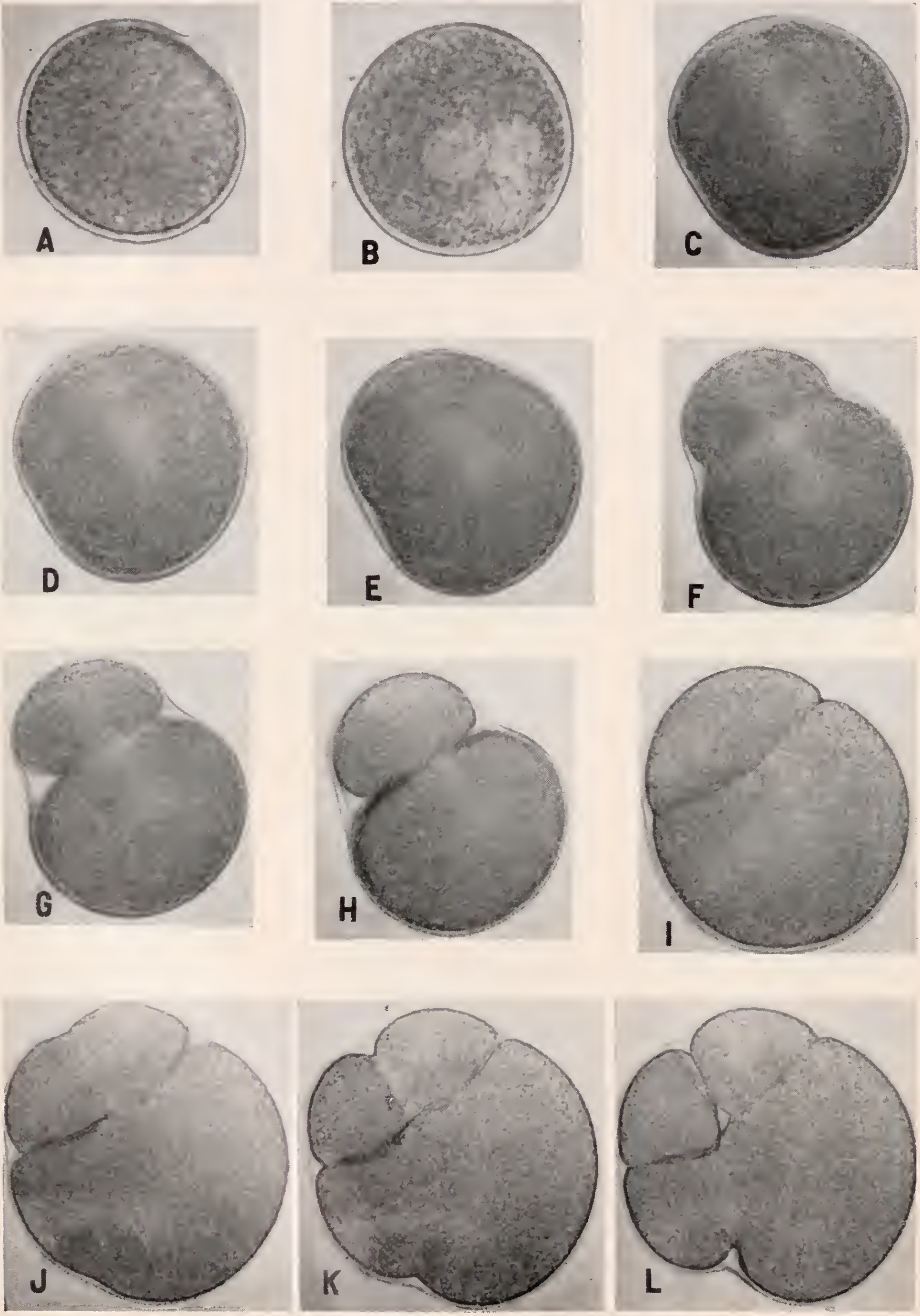
Formulas for approximately 4 liters of artificial sea water:

Substance	Sea water	Ca-Free	K-Free	Mg-Free
NaCl	93.90 grams	93.90	93.90	93.90
$MgCl_2$ $(6H_2O)$	42.35	42.35	42.35	—
Na_2SO_4	15.65	15.65	15.65	15.65
$CaCl_2$ $(2H_2O)$	6.10	—	6.10	6.10
KCl	2.65	2.65	—	2.65
$NaHCO_3$	0.76	0.76	0.76	0.76
Water	4076.0	3951.0	4003.0	3253.0

Isotonic solutions for Spisula eggs (from measurements of egg diameters):

Substance	Molar concentration
NaCl	0.50
KCl	0.52
$CaCl_2(2H_2O)$	0.35
$MgCl_2(6H_2O)$	0.34
Sucrose	0.73 (approximate)
Urea	0.96
Na citrate	0.35

Plate II. *Spisula solidissima* eggs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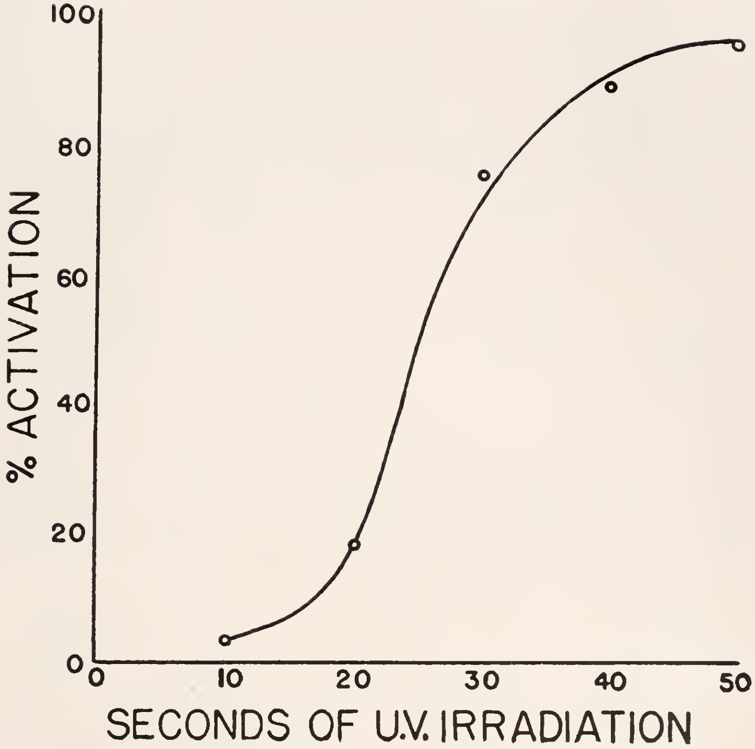

FIGURE 1. A typical population curve for a lot of eggs, obtained by plotting excitability against duration of exposure to the stimulating agent.

A sea water mixture containing only the chloride salts can be made by mixing isotonic chloride solutions in the following proportions: NaCl, 100 parts; KCl, 2.65; $CaCl_2$, 2.42; $MgCl_2$, 12.10.

RESULTS OF EXPERIMENTS

A. Artificial activation

Methods of activation were standardized to make possible a study of the influence on excitation of separate environmental factors and other agents. For this purpose curves were plotted for percentage of activation as a function of exposure time to the stimulating agent (usually ultraviolet light). The curve obtained in all cases was essentially one representing the distribution of excitabilities of individual eggs of a population. The shape and slope of the curve varied to some extent

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A. Egg 43 minutes after insemination; note polar and clear region containing the spindle remnant.

B. The same egg 51 minutes after insemination; showing both pronuclei.

C–H. Photographs of a single egg at 21° C. taken at half-minute intervals from 71 minutes (C) to 73½ minutes (H).

I–L. Photographs taken of the same egg slightly compressed by a cover slide in order to show cleavage details. The time intervals between photographs was one-half minute, beginning at 93 minutes (I) and ending at 94½ minutes (L).

TABLE I

Artificial activation in Spisula eggs

Type of stimulation	Dose or concentration	Exposure time	No. of experiments	Maximum % activation	Reproducibility	Character of shape changes induced
U.V. light	4500 μWatts/cm.^{2}	15 sec.–1 min.	116	100	excellent	Slight wrinkles at surface
Excess K$^+$	4–6% KCl-SW	4+ min.	69	100	good	Slight wrinkles at surface
K-Free SW	100%	1–4 min.	8	100	good	Slight wrinkles at surface
NH_3	10^{-4}–10^{-3} *M*	1–4 min.	12	100	good	Slight wrinkles at surface
Hypertonicity	100 ml. SW + 30–50 ml. 2.5 N NaCl	1–5 min.	15	100	fair	Deep indentations into the cytoplasm
Hypotonicity	10–20% SW	1–3 min.	78	100	good	Deep indentations on return to sea water
Heat	25–40° C.	10 sec.–15 min.	8	10	poor	Deep indentations (prolonged but reversible)
Cold	0–5° C.	10 sec.–15 min.	4	10	poor	Deep indentations
Urea	1–100% isotonic urea-SW	1–10 min.	10	5	poor	Sudden deep indentations
Protamine	50–200 mg. %-SW	30 min.–4 hrs.	6	100	fair	Shallow indentations

among different lots. Whenever difficulty was encountered in obtaining 95–100% activation by artificial agents, fertilization of the lot was usually found to be equally poor. Figure 1 shows an example of such a threshold curve for a lot of eggs.

A number of agents which have been employed in artificial parthenogenesis of various marine eggs will activate the eggs of *Spisula* (Table I). In most cases, it

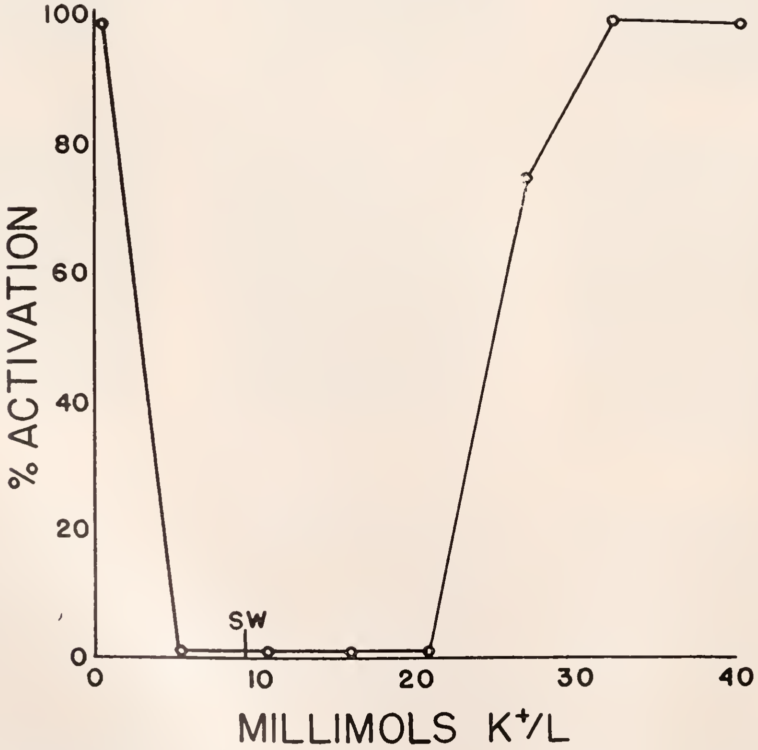

FIGURE 2. The activating effect of potassium ions above and below the concentration found in normal sea water.

was found possible to adjust the concentration of the activating agent so that only short exposure times were required for complete or nearly complete activation. Only in the case of excess potassium ions and of protamine (clupein) was it necessary to expose for longer than one to three minutes in order to obtain a result. Of the agents listed in Table I, only heat, cold and urea were distinctly unsatisfactory as activating agents for *Spisula* eggs. They are listed, however, for their interesting property of causing pronounced changes of shape in the eggs.

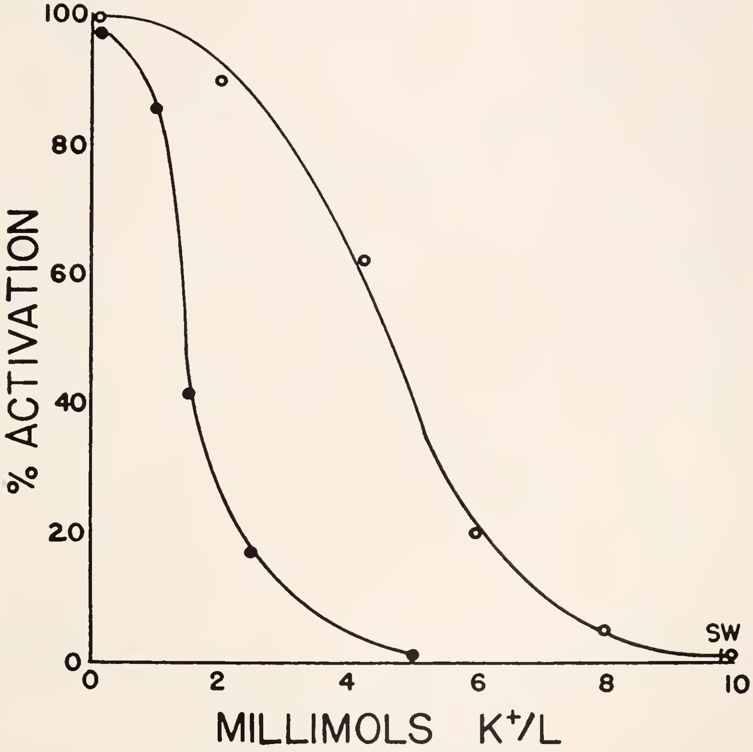

FIGURE 3. Curves showing (o) synergistic effect of low potassium concentrations in sea water and sub-threshold ultraviolet light stimulation, and (●) the activating effect of low potassium concentrations alone.

B. Changes in excitability following alteration of the medium

Experiments were carried out to determine the influence of changes in the environment on excitation, using an excitability curve (such as Fig. 1) as a guide to dosage. The concentration of each ion (except sodium) was varied while keeping tonicity and pH constant.

It was important to know how long eggs must remain in contact with a new medium before a change in excitability is manifested. The new environment causes the same degree of effect on the response whether the eggs are placed in it before, or up to four minutes after application of the activating agent. For this reason, in the experiments reported here, eggs were first stimulated by a dosage of ultraviolet light (determined by a threshold curve) and then immediately transferred to separate dishes containing different amounts of the ion or other substance under consideration.

Even small increases or decreases in potassium concentration cause increased excitability. Both excess potassium and potassium-free sea water are excellent activating agents (Fig. 2). Figure 3 shows the effect of low potassium concentrations alone and in synergism with ultraviolet light.

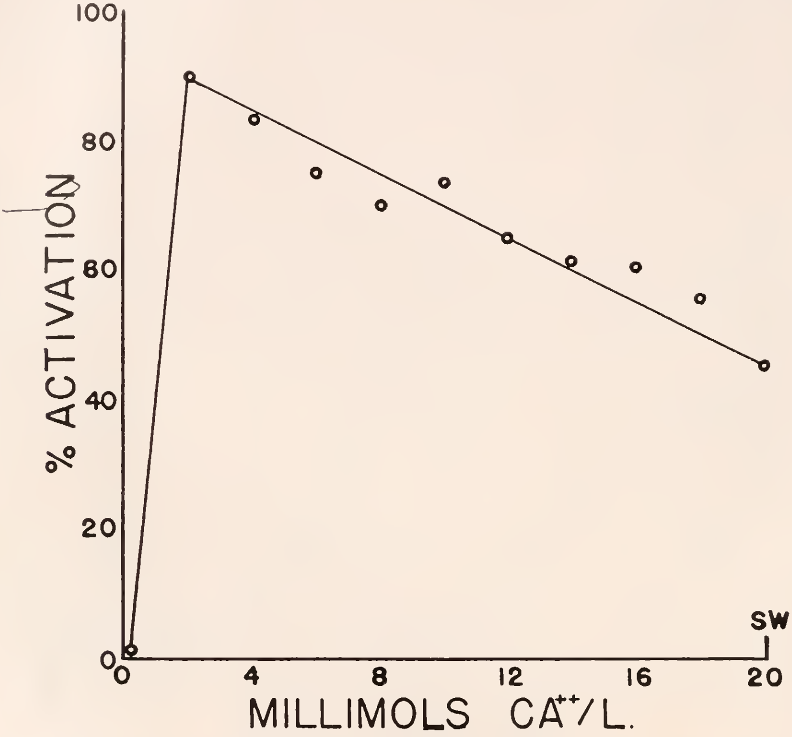

FIGURE 4. The effect of reduced calcium concentration on excitability (ultraviolet light stimulation). Dose of irradiation chosen to give 50% activation in normal sea water.

It was not possible to demonstrate an activating effect of any concentration of calcium. Calcium, however, apparently does enter and leave the egg, at least the cortical region, because even a slight alteration in calcium content of the medium causes a change in threshold to ultraviolet light stimulation (Fig. 4). Decreasing the calcium concentration results in a marked increase in excitability; a minimum amount of calcium is required for any excitation, however (about 5×10^{-4} *M*). Excess calcium ions reduce excitability. These effects are reversible.

If irradiation of the eggs by ultraviolet light in the absence of calcium is followed by transfer of the eggs into normal sea water, activation results. The percentage of activation is an inverse function of the time elapsed between the onset of irradiation and the transfer to the calcium-containing medium (Fig. 5). Other activating agents failed to give activation under these circumstances.

The effects produced by magnesium are quite similar to those of calcium as far as threshold is concerned. However, magnesium is apparently not necessary for activation. Figure 6 shows that excitability is high at low concentrations of magnesium and low when excess magnesium has been added.

Fertilization seems to be unaffected by changes in pH between 7 and 9 under conditions where ample sperm are present. At pH values below 7, the percentage of eggs activated by the sperm decreases rapidly. Below pH 5.0–5.2 there is usually no activation (Fig. 7). Using a strength of ultraviolet stimulation which at pH 8.0–8.2 activates 95–100% of the eggs, it is found that the pH at which total inhibition occurs is about the same as that at which fertilization is inhibited; see Figures 7 and 8. However, it appears that the shape of the pH-dependency curve differs for the three kinds of activation (sperm, ultraviolet light, and osmotic stimuli).

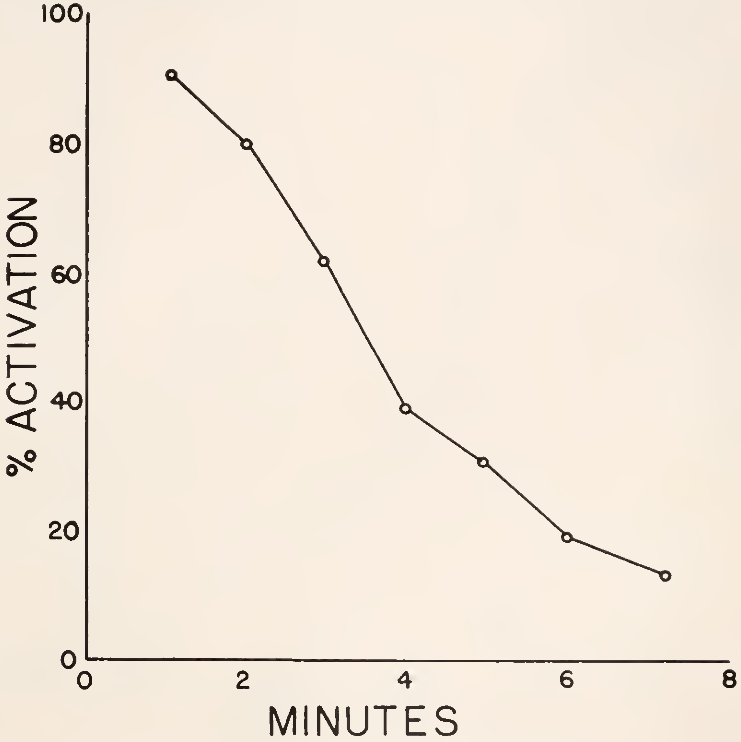

Figure 5. Activation obtained if eggs are irradiated in calcium-free sea water and subsequently transferred to normal sea water at the times indicated. Dose of irradiation chosen to give 100% activation in normal sea water.

Since a rise in temperature accelerates the rate of many chemical reactions, it might be expected that heat would accelerate whatever reactions may be involved in excitation. In *Spisula* eggs, however, the opposite seems to be true; eggs stimulated at room temperature exhibit higher percentages of activation if transferred to sea water of lower temperature (Fig. 9). Similarly, eggs stimulated at low temperatures are inhibited if transferred to higher temperatures. On the other hand, previous exposure to heat (30–35° C.) or cold (0–5° C.) for a few minutes increases excitability when eggs are subsequently stimulated by ultraviolet light. It should be emphasized that heat and cold by themselves are not successful activating agents for *Spisula* eggs. These agents do, however, influence the response mechanism, as evidenced by their effects on the shape of the eggs and on excitability.

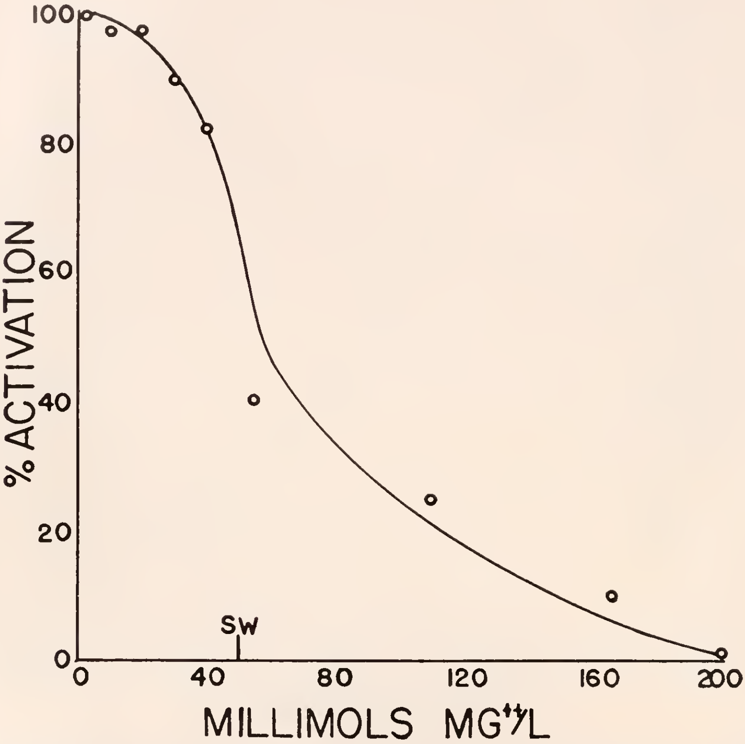

Figure 6. The influence of reduced and increased magnesium concentration on excitability. Dose of ultraviolet light chosen to give 50% activation in normal sea water.

The anesthetics ethyl ether (Fig. 10) and ethyl urethane (Fig. 11) inhibit activation by sperm and decrease excitability for artificial activating agents. Chloroform causes extensive injury even at relatively low concentrations and therefore is not a good inhibitor for *Spisula* eggs. The egg jelly extracted with acid sea water and neutralized also exhibits a weak inhibitory influence on activation in sufficiently high concentrations.

Although normal fertilization causes only a slight and gradual membrane elevation, ultraviolet light in synergism with sea water of altered ionic constitution often causes a more pronounced elevation of the membranes. The monovalent cations are especially potent in this respect.

C. Duration of sensitivity to inhibitors

Fertilization in the presence of calcium could be reversed during the first four or five minutes of development by immersing the eggs in any of the following media: *a*, 10–20% isotonic sodium citrate[4] calcium-free sea water; *b*, acidified sea water (pH 5; made by adding 0.5 N HCl to unbuffered sea water), or *c*, 0.3–0.5% (by volume) ether in sea water. Developmental changes initiated during the first four or five minutes after fertilization could be reversed, because many of these eggs could be returned to normal sea water and activated a second time, this time by an artificial activating agent. These reactivated eggs often cleaved, in contrast

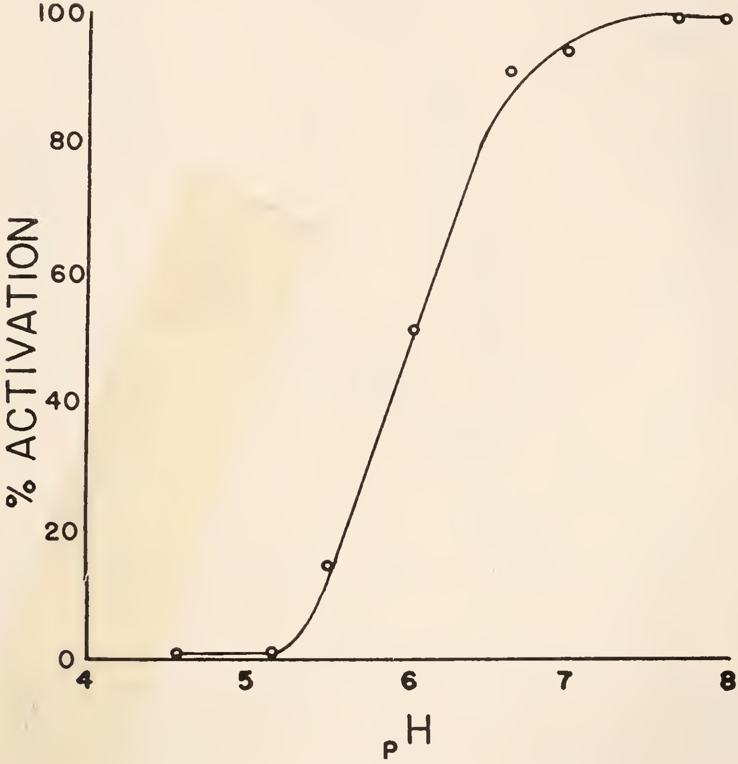

FIGURE 7. The influence of pH on fertilization (pH adjusted by adding HCl to bicarbonate-free sea water).

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[4] Citrate is necessary to take care of the calcium carried over with the eggs; this concentration is well below the toxic level.

to the artificially activated ones which almost never cleave. Figure 12 defines the term "Stage I" which is the period during which eggs activated in normal sea water are sensitive to arrest by lack of calcium, lowered pH, or the presence of ether in their final medium. The time remaining until germinal vesicle breakdown is designated as "Stage II."

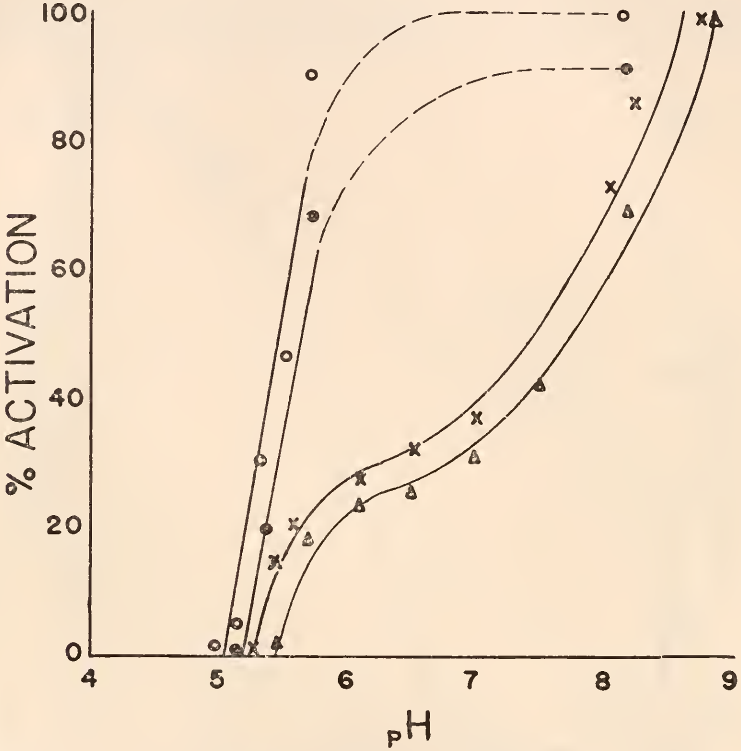

FIGURE 8. The influence of pH on artificial activation (pH adjusted by adding HCl to bicarbonate-free sea water). (o) represents 45 secs. ultraviolet irradiation; (●) represents 35 secs. irradiation; (×) represents hypotonicity, (△) represents hypertonicity. Dosages of stimulating agents arranged to give 90–100% activation at normal sea water pH.

D. Changes during Stage I

As mentioned earlier, slight changes of shape are often observed following sperm penetration or application of activating agents. Surface wrinkling or small indentations are often seen after application of some agents and deep indentations after others (*cf.* Tables I, II; Plate I). Artificial activating agents often evoke changes of shape more exaggerated than those resulting from fertilization. Eggs which are stimulated by ultraviolet light show marked wrinkling at the surface; sodium ions, isotonic urea (alone or in mixtures with sea water), heat and cold produce deep indentations which last from seconds to minutes. Such changes in shape cannot be induced in the absence of calcium. Departure from spherical shape can be associated with changes in volume. In haematocrit experiments it was found that a decrease of at least 6–8% in volume follows stimulation by heat, urea, and sodium chloride if calcium is present. Decrease in volume means expulsion of water from the protoplasmic gel (syneresis). Such a gelation was detected by the centrifuge method after heat stimulation, which treatment also caused the most prolonged indentations. The brevity of response to other agents made similar viscosity measurements by this method impossible. (Viscosity measurements are very difficult in unfertilized *Spisula* eggs because forces of the order of 200,000 times gravity are required to stratify the particulate matter in the cytoplasm.) There is a rapid increase in the rate of brownian movement at the end of Stage I; It is at about this time that the cell regains its spherical shape, probably through the uptake of water (imbibition).

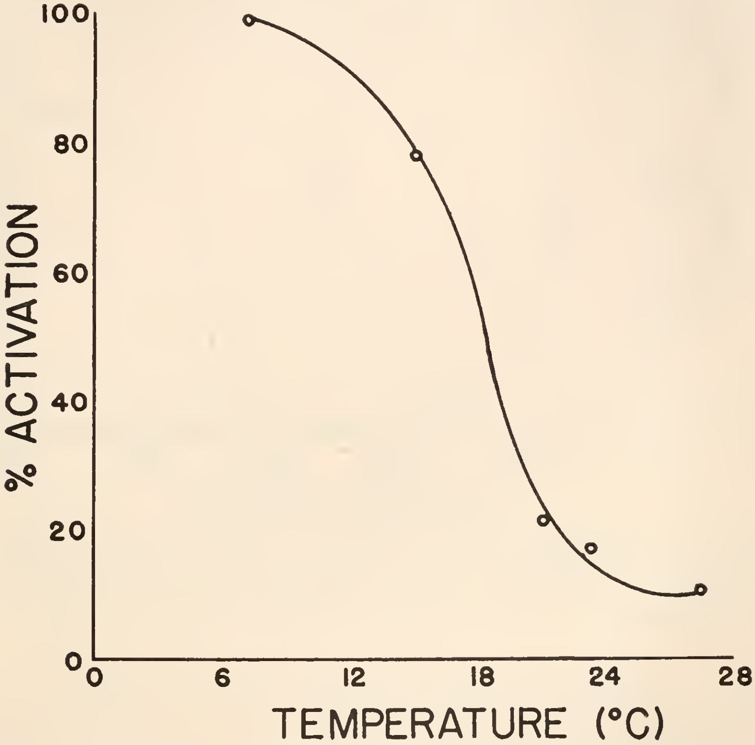

FIGURE 9. The effect of reduced temperature on excitability. The temperature of stimulation was 27° C. The dosage of ultraviolet light was chosen to give about 10% activation at this temperature. Aliquots of the stimulated eggs were transferred immediately to sea water at a lower temperature.

The application of mixtures of two or three of the sea water cations in the proportions found in natural sea water shows some effects of ion antagonism on

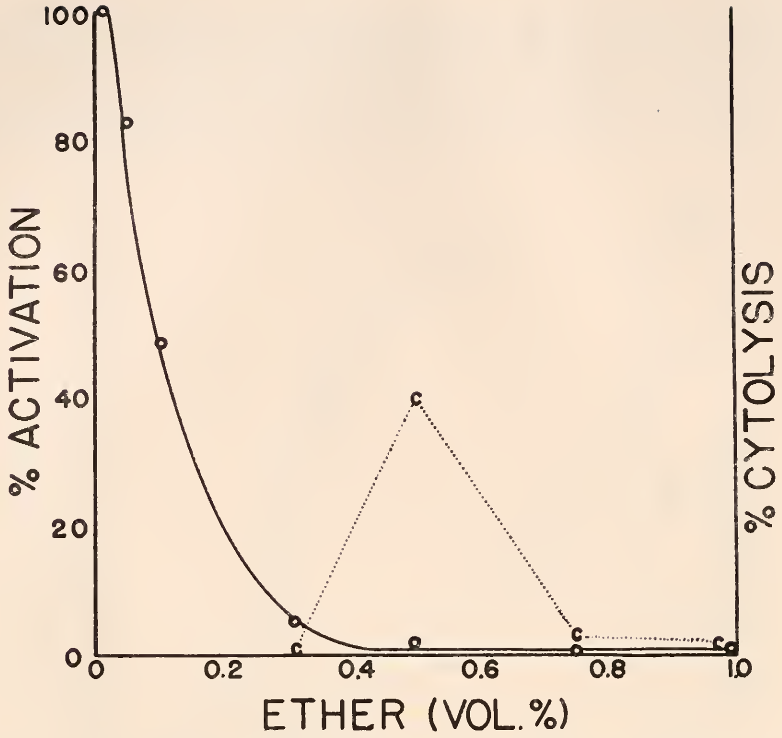

FIGURE 10. The inhibiting effect of ethyl ether. Eggs in sea water stimulated by dosage of ultraviolet light chosen to give 100% activation and subsequently transferred to sea water containing ether. The dotted line represents percentage of cytolysis (plotted on the same numerical ordinate).

TABLE II

Ion antagonism associated with changes of shape on stimulation*

Na^+	K^+	Ca^{++}	Mg^{++}	Wrinkling	Indentations
+	−	−	−	Marked	Few
+	+	−	−	Very slight	None
+	−	+	−	Marked	Marked
+	−	−	+	Slight	None
+	+	+	−	Few slight	None
+	+	−	+	None	None
+	−	+	+	Slight	None
+	+	+	+	None	None
−	+	+	−	None	None
−	+	+	+	Slight, prolonged	None
−	+	−	+	None	None
−	−	+	−	None	None

* Eggs were transferred with a minimum of sea water to mixtures of the isotonic cation chlorides. Thus in these mixtures, traces of all of the sea water cations were present. In the case of calcium, sufficient quantities of this ion were present to permit activation or changes of shape. The further addition of calcium intensified the shape changes.

changes of shape induced by sodium ions. Sodium ions in the presence of traces of calcium elicit sudden strong indentations. Table II shows that an excess of calcium intensifies the magnitude of the sodium response as long as the other cations are absent. Both potassium and magnesium alone weaken this response, and the two together abolish it. This is probably the reason that eggs do not normally show indentations when placed in sea water. It is true that freshly shed eggs are irregular in outline; this seems to be due to previous compression in the ovary. Hydrogen ions tend to prolong indentations caused by activating agents. Ether will not prevent indentations even though it inhibits germinal vesicle breakdown.

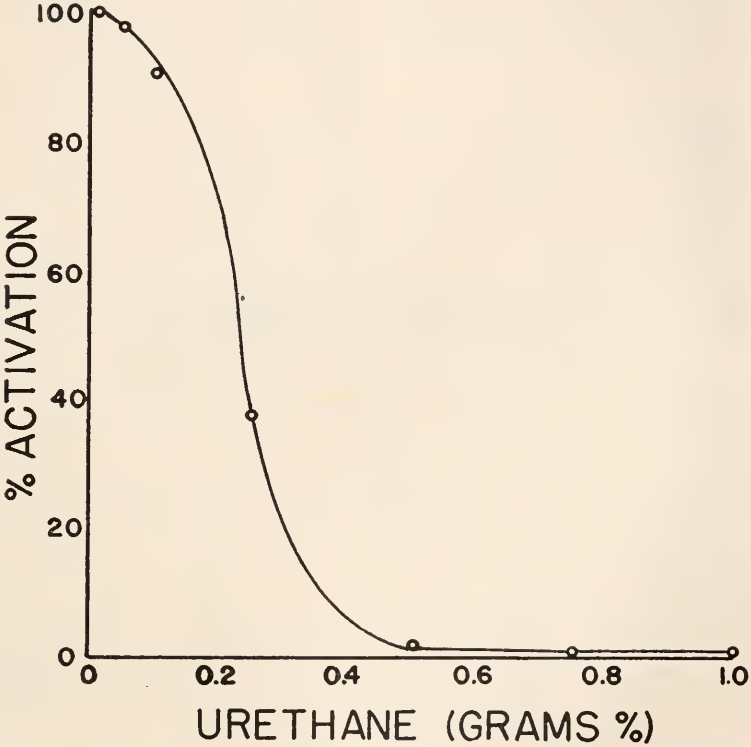

FIGURE 11. The inhibiting effect of ethyl urethane on stimulation by ultraviolet light. Dose of irradiation chosen to give 100% activation.

In fact, higher concentrations of ether can induce deep indentations. In living eggs, the removal of calcium by 10–20% sodium citrate in calcium-free sea water is the only way to prevent changes of shape following stimulation; this inhibition is reversible. Strong poisons may act similarly in preventing changes of shape, but their effects are not reversible. Thus iodoacetate (4×10^{-4} *M* but not 3×10^{-4} *M*) will inhibit changes of shape. Cyanide (10^{-3} *M*), or bromide (10^{-2} *M*) have no detectible inhibitory effect. Attempts at causing indentations with sodium ATP (up to 10^{-2} *M*) failed. A series of experiments was performed in which eggs were transferred first to dilute sea water and then to various stimulating solutions of the same dilution (osmotic strength). Such a transfer of eggs from sea water to 50% sea water, and then to 50% isotonic urea resulted in surface indentations. Below this dilution, indentations were not detected. In another experiment indentations were observed following transfer from 35% sea water to 35% isotonic sodium chloride. In all of these experiments, and those summarized in Table II, the traces of calcium which accompanied the eggs when they were transferred from sea water to the test solutions were sufficient to permit changes of shape, and in some cases, nuclear breakdown.

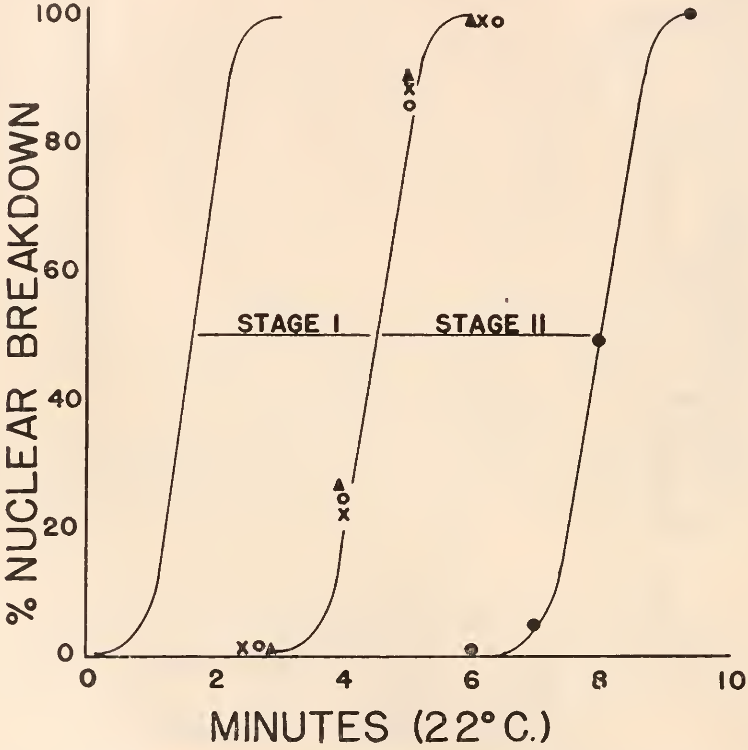

Figure 12. The duration of sensitivity to lack of calcium, acid sea water, or ether in eggs activated in normal sea water and subsequently transferred to these inhibitors. The right-hand curve represents the time at which dissolution of the germinal vesicle membranes is observed following fertilization. The left-hand curve is a hypothetical curve of the same slope, representing the probable penetration times of the sperm. The middle curve represents the time at which the fertilized eggs lose their sensitivity to inhibition by lack of calcium (o), acid sea water (×) and ether (▲). The time differences between the curves are called "Stage I" and "Stage II" as indicated in the figure.

During the course of Stage I there is measurable acid production in fertilized or artifically activated eggs. This is especially noticeable when eggs are suspended in unbuffered, bicarbonate-free sea water.

Discussion

A. Changes at the surface of the egg following activation

In the eggs of many marine invertebrates the penetration of the sperm causes striking changes at the cell surface. Although eggs of the sea urchin and other echinoderms exhibit highly elevated fertilization membranes, in general, the eggs of annelids and molluscs do not. There may be slight membrane elevation in these forms, however. Furthermore, in *Spisula* eggs, the cortical granules do not appear to be altered by fertilization or by artificial activation as they are in the sea urchin (Moser, 1939a, 1939b; Runnström, 1949), except perhaps under very severe conditions of artificial activation by ultraviolet light in synergism with an excess of one of the sea water cations. Under these conditions, widely elevated membranes are sometimes seen, and it is difficult to tell whether the cortical granules are changed or not.

The surface, especially the cortex of the egg, is important during the initial response of the egg to activating agents. Evidence for the importance of the cortex has been presented by Whitaker (1931), Runnström (1923), Monroy and Montalenti (1947) and by Wilson and Heilbrunn (1952). Smith and Clowes (1924), and Tyler and Schultz (1932) showed that a number of marine eggs could cleave normally at hydrogen ion concentrations greater than required to inhibit fertilization. This suggests that at least part of the fertilization reactions take place at the surface. Runnström and Kriszat (1952) believe that the cortex plays a "master role" in initiating a "chain reaction" at fertilization.

Some information concerning the role of the cortex can be inferred from the effects of various environmental changes on excitability. According to current opinion, the cortex is probably the only part of the cell into which ions from the environment can freely diffuse (*cf.* Mazia, 1940). Since minor changes in the ionic constitution of the sea water environment have an immediate and maximal effect on excitability, it seems logical to suppose that the part of the cell primarily involved in response reactions has come into a dynamic equilibrium with the new environment. It seems probable that these minor ionic changes must be acting primarily on the cortex to produce their effect so rapidly and drastically. Furthermore, minor changes in ionic environment which cause significant excitability changes have no detectible effect on cell division or later development. The fact that slight alterations in ionic balance tend to act synergistically with ultraviolet light to cause widely elevated membranes to appear indicates that these alterations do bring about cortical changes. That cell division proceeds in sea water modified in this way is evidence that the interior of the cell is not seriously affected. Thus the data on excitability changes under altered environmental conditions can be interpreted in terms of an effect on the cortex. These changes may well be related to the changes in cortical viscosity such as those described by Wilson and Heilbrunn (1952). However, other factors may be affected as well.

It is believed that, generally, the internal hydrogen ion concentration of a cell is maintained relatively constant over a wide range of external pH. Tyler and Schultz (1932) showed that fertilization was particularly sensitive to slightly acid sea water (pH 6.8–7.5). The excitability of *Spisula* eggs varied with external hydrogen ion concentration (Figs. 7 and 8). Accordingly, the curves of acid inhibition of activation could be interpreted as inactivation curves of some surface enzymes, or processes occurring at the surface. Another possibility is that acid is affecting the dissociation of calcium from its protein binding, thereby disturbing the normal release of calcium following stimulation. Such a release of calcium could conceivably pass around the surface of the egg, as Moser (1939a, 1939b) believed, in a chain reaction similar to that postulated by Runnström and Kriszat (1952). However, the different curves of acid inhibition obtained with ultraviolet light on the one hand, and with osmotic stimulation on the other, might indicate somewhat different pathways for the response mechanism for these different stimulating agents, and for fertilization as well.

It has been shown (Fig. 2) that a lack of an excess of potassium in the normal environment can cause activation. Activation by potassium-free sea water is actually sodium activation. Although sodium and potassium ions both stimulate, they act antagonistically toward the cell. It may be primarily this antagonism which keeps the *Spisula* egg in the germinal vesicle stage until it is fertilized, but it is hard to imagine how the sperm or other stimuli could exert an effect by influencing this kind of a block, unless by a permeability change, for which there is at present no evidence in the *Spisula* egg. A similar situation of sodium-potassium antagonism appears to exist in the eggs of *Urechis* (Scheer and Scheer, 1947). Other factors, especially the divalent ions, pH and temperature doubtless also contribute to block activation. Wilson and Heilbrunn (1952) believe that either sodium or potassium can release calcium from its binding with protein in the cortex. Why these ions both stimulate (*cf.* results of Sawada, 1952, on *Mactra veneriformis*) and yet act antagonistically is not known. Sodium and potassium seem to act differently in their ability to initiate development (Allen, 1952b). The time required for nuclear breakdown is the same if eggs are activated by potassium (sea water with double potassium concentration) as if they are activated by sodium (potassium-free sea water). This indicates that developmental processes are initiated at the same time in both media. Eggs activated by sodium can be removed after a minute and developmental changes continue in sea water; eggs activated by potassium, however, must remain in the stimulating solution throughout Stage I (four to five minutes). If they are removed during this period and placed in sea water, the developmental processes already initiated will become reversed, as if they had been placed in an inhibitor.

Calcium plays an important role in the excitation of marine eggs and other cells. The necessity of calcium for fertilization and artificial activation is well known (see especially Loeb, 1915; Dalcq, 1928b; Pasteels, 1935). Of the parthenogenetic agents tried by Wison and Heilbrunn (1952), calcium was the only one which caused the cortex to stiffen. As a parthenogenetic agent, calcium is unusual in the length of exposure it requires to be effective. The results of this study (see Fig. 4) indicate that calcium enters the cortex and causes a decrease in excitability. Similarly, if calcium is withdrawn from the environment (and as a result, from the cell), excitability is increased. For many eggs, including *Spisula,* calcium is a poor activating agent. The parthenogenetic action of calcium in some eggs may be due to a leakage through the cortex; for, once within the cortex this calcium may imitate the effect of normal release of calcium by stimulating agents. The effects of calcium on excitability can be explained by postulating that agents which liquefy the cortex (*cf.* Wilson and Heilbrunn, 1952) act synergistically with one another and antagonistically with those agents, such as calcium, which tend to stiffen the cortex. In this way, it would be possible to account for the action of the cations on excitability.

Magnesium acts as an anesthetic in *Spisula* eggs as in other cells (Wilbur, 1939; Sawada, 1952). Its effect on cortical viscosity has not been determined in marine eggs.

Ether is a good anesthetic for *Spisula* eggs and works best on activation when its concentration is below 0.3% (by volume). At slightly higher concentrations (about 0.5%) ether causes wide-spread cytolysis. At still higher concentrations this cytolysis is prevented, but the eggs will now cytolyze if returned to normal sea water. It is possible that ether causes cytolysis by some process which resembles stimulation, such as a release of calcium or an increase in permeability. The prevention of cytolysis at higher concentrations may be a direct inhibition of the enzymes responsible for disintegration of the cell.

The fact that activation in *Spisula* eggs is favored by reduced temperature (Fig. 9) does not support the theory of R. S. Lillie (1941 and earlier) for activation of starfish eggs. Much of the evidence gathered in this investigation is in opposition to Lillie's theory. Activation by some agents is so rapid in *Spisula* eggs that it is difficult to conceive of the formation of a complex activating substance such as Lillie postulated.

According to the colloidal theory of stimulation proposed by Heilbrunn and his students, all stimulating agents in general act on the same series of processes, namely, those which are involved in, or follow the release of calcium from protein binding in the cortex. Presumably the normal release of calcium would be brought about by a change in the binding capacity of the cortical proteins. An attempt was made to demonstrate that the binding capacity of these cortical proteins could be influenced by stimulating agents presented in the absence of calcium, with the result that subsequently added calcium would be able to initiate development. This was accomplished by ultraviolet stimulation (Fig. 5). Apparently the eggs recover from such ultraviolet effects, as evidenced by the decrease with time of their ability to respond to added calcium. Atttempts to duplicate these results with other activating agents failed.

The fact that egg excitability could be influenced after withdrawal of the stimulating agent, but only during the subsequent 4–5 minutes (Stage I), is apparently related to the fact that eggs are sensitive during this period to arrest by inhibitors (Fig. 12). Inhibition by acid at this stage suggests that a surface reaction is affected. Simultaneous susceptibility to lack of calcium suggests that this surface process requires calcium. It appears probable that the environmental changes and other agents which affect excitability do so by their influence on these surface processes occurring during Stage I The presence of such an inhibitor-sensitive period following fertilization was first shown by Tyler and Schultz (1932). Similar experiments were performed by Goldstein (1953) on the activation (spontaneous maturation) of the *Chaetopterus* egg. Goldstein found two stages which were inhibited by lack of calcium and by carbon dioxide, respectively; however, this maturation process is not inhibited by acid sea water (above pH 3.5), whereas fertilization is inhibited at a much higher pH (7.1) in this egg (Smith and Clowes, 1924).

Although from much indirect evidence it would be tempting to believe that all stimulating agents work by initiating the same sort of response reactions, the following evidence seems to suggest that there may be variations in pathway to different agents: (1) the pH-dependency curves for activation by different agents seem to be dissimilar (Figs. 7, 8); (2) sodium and potassium, which act antagonistically in sea water, each cause activation when presented in excess or not antagonized. Furthermore, those ions seem to influence the activation processes differently. (3) Activation can be obtained if eggs are irradiated by ultraviolet light in the absence of calcium and subsequently transferred to normal sea water. These results could not be duplicated with other activating agents.

II. Changes in the interior of the egg following activation

Following fertilization, chemical changes in the interior cytoplasm and nucleus prepare the egg for its metabolic needs during development (for discussion, see Runnström, 1949; Brachet, 1950). Colloidal changes in the cell interior probably also play a role in the processes leading to cleavage and further development (see Runnström, 1949; Heilbrunn, 1952). The fact that calcium is required for changes of shape (see Table II) caused by stimulation indicates that this ion is involved in these colloidal changes. For reasons mentioned earlier, it was not possible to obtain direct information concerning cytoplasmic viscosity changes at activation. The resistance of *Spisula* eggs to stratification by centrifugal force appears to be due mostly to their particularly high viscosity. However, this resistance to stratification might also be due in part to a lack of any marked difference in specific gravity between the granules and the ground substance. This possibility was indicated by the fact that the fat-containing granules collect somewhat more rapidly at the centripetal pole than do the heavier granules at the centrifugal pole when the eggs are subjected to forces of about 200,000 × gravity for from one to three minutes. The fact that the cytoplasm is a gel is also indicated by the formation of a ring of negative strain birefringence around the nucleus when this structure is moved through the cell by centrifugal force.

Changes in shape (indentations) similar to those found in the present investigation were described in *Spisula* eggs by Schechter (1941) in connection with natural changes which occur on aging. He showed that these changes were brought about more rapidly when calcium was present in the medium. It is thus interesting that calcium is associated with the occurrence of indentations as a result of both aging and stimulation.

It has been pointed out above that the changes of shape observed are actually a visible expression of syneresis (loss of water) by the egg protoplasm. Some similar changes in shape were reported by Tyler (1932) in the egg of the echiuroid worm, *Urechis caupo*. This egg already possesses a polar indentation before fertilization. The indentation disappears, reappears, and disappears for a second time before the germinal vesicle breaks down. Tyler made measurements to show that volume changes are involved, and that as the first indentation disappears, there is a drop in viscosity. Possibly the second indentation in *Urechis* is similar to the indentation observed in the present study. Tyler explained the changes in shape and volume by assuming changes in internal osmotic pressure. Since viscosity changes seem to have been involved as well, syneresis and imbibition may have been the immediate factors involved in the shape and volume changes. Presumably, the forces developed by a contracting gel could oppose those of osmotic pressure. This certainly is true in the *Spisula* egg, as mentioned earlier in the results, where deep indentations occur even when the stimulating media are diluted with distilled water. Contractions or wrinkling at the surface of eggs seem to be of rather general occurrence (for example, see Runnström, 1949).

The loss of water by the egg is followed after a few minutes by a return of water (imbibition) and a rounding up of the egg's contour. This entrance of water causes a sharp increase in the rate of brownian movement, indicating liquefaction. It is at about this time that the processes designated as Stage I come to an end.

The rapidity with which eggs undergo cytolysis with disintegration following excessive stimulation is an indication of the relationship of stimulation to the activation of enzymes in the egg (for discussion of this point, see Runnström, 1949). Goldstein (1953) who has studied the maturation process in the eggs of the annelid worm, *Chaetopterus,* suggests that the nuclear membrane is dissolved by a calcium-activated proteolytic enzyme. There is ample evidence for the presence of proteolytic enzymes in the egg cell after fertilization (Lundblad, 1944; Woodward, 1950; Gross, 1952). It has been observed (Allen, 1951b) that in several marine eggs, the breakdown of the germinal vesicle is preceded by dissolution of the nucleolar membrane. It is thus possible that if an enzyme is involved in nuclear and nucleolar breakdown, it may come from within the nucleus. Since such an enzyme would presumably be calcium-activated (because some calcium is necessary for nuclear breakdown) it could, as far as is now known about calcium-activated enzymes, be either proteolytic, as Goldstein suggests, or lipolytic.

SUMMARY

1. The present study is a preliminary survey of fertilization and artificial activation in the egg of the surf-clam, *Spisula solidissima* (Dillwyn).

2. The structure of the egg, optimal conditions for fertilization, and normal early development of fertilized and artificially activated eggs are described.

3. The results of treatment by various parthenogenetic agents are discussed with particular reference to possible similarities and differences in their mode of action and pattern of response initiated. Among the agents discussed are: ultraviolet irradiation, potassium, sodium, ammonia, osmotic stimuli, heat, cold, urea, and protamine (clupein).

4. The influence of various changes in environmental conditions has been investigated and the results correlated with the function of the cortical region of the cytoplasm during the first four or five minutes immediately following stimulation. Monovalent cations, temperature shock, and stimulation in the cold all increase excitability. Divalent ions, stimulation at slightly elevated temperatures, lowered pH, or the addition of ether, urethane or egg jelly decrease excitability.

5. Sodium-potassium antagonism may be largely responsible for maintenance of the egg in the germinal vesicle stage prior to fertilization.

6. The changes which can be detected in the interior cytoplasm and in the nucleus following activation are discussed with particular reference to changes of shape and volume, and to nuclear breakdown.

7. It is concluded that shape changes are probably caused by an expulsion of water (syneresis) from the cytoplasmic gel when the egg is activated. This expul-

sion of water is probably due to an increased gelation caused by a release of calcium from the cortex by activating agents.

8. The response mechanism of the egg can be divided into two stages on the basis of the fact that for 4–5 minutes after activation it is susceptible to inhibition by acid sea water, by lack of calcium, or by dilute ether; but after this time inhibition by these agents is no longer possible.

9. Evidence concerning the direct cause of germinal vesicle breakdown is discussed, and this evidence suggests that a calcium-activated proteolytic or lipolytic enzyme is involved.

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TISSUE RESPIRATION, GROWTH, AND BASAL METABOLISM

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It is a phenomenon general in mammals and other classes of the animal kingdom that metabolic rate per unit weight decreases with increasing body size. This is expressed in the surface rule of Rubner who stated that metabolic rate decreases per unit weight, but is constant per unit surface. More recent investigations (Brody, 1945; Kleiber, 1947) indicate that interspecifically, *i.e.,* comparing mature animals of different species, basal metabolic rate in mammals is proportional rather to a ¾ power of weight than to surface or the ⅔ power of weight. Intraspecifically, *i.e.,* comparing animals of different body size within the same species, the surface rule applies to the general trend of the size-metabolism relation in rats, although qualifications have to be made in detail (Bertalanffy, Müller and Racine, unpublished data). These complications, however, do not alter the fundamental fact of the decrease in weight-specific metabolic rate with increasing body size. However, we do not have a satisfactory explanation for this phenomenon.

The basic alternative seems to be whether the dependence of metabolism on body size is based upon *cellular* or *organismic* factors. It may be due to intrinsic differences in the metabolism of the cells of smaller and larger individuals which will show up also in isolated tissues; or it may be due to regulative factors lying in the organism as a whole. There may be also a combination of both.

Earlier work on the relation between tissue metabolism and body size (reviewed by Kleiber, 1947) is contradictory. Terroine and Roche (1925), and, independently, Grafe (1925; Grafe, Reinwein and Singer, 1925), stated that the metabolic rate per unit weight of homologous tissues *in vitro* is essentially the same for small and large animals, although basal metabolic rate per unit weight *in vivo* decreases systematically with increasing body size. Grafe assumed that the metabolic rate of tissue *in situ* is checked by central regulators, mainly the nervous and endocrine system. On the other hand, LeBreton and Kayser (1926; Kayser, LeBreton and Schaeffer, 1925), and Borger and Groll (1926) reported variation of the respiration rate of tissues with increasing body size, in individuals of the same species as well as in different species. This earlier work is open to criticism, and Grafe *et al.'s* calculations, in particular, were based upon erroneous assumptions (*cf.* Field *et al.,* 1939).

More recent results, however, are also contradictory. According to Field *et al.* (1939) the summated tissue respiration (*i.e.,* metabolic rate *in vitro* per unit fresh weight, multiplied by the weight of the respective organ, and summated over 20 main organs) amounts to 66% of the basal metabolic rate of the rat, and, if allowance is made for minimal functional activity (muscle tone, cardiac, respiratory, smooth muscle, secretory function), even for 89% of the respiration of the intact animal. The authors conclude that basal metabolism is the arithmetic sum

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of tissue respiration as measured by the Warburg technique, and that there is no reason to assume that the level of tissue respiration *in situ* is determined by organismic factors which are not operative *in vitro*. Similar experiments were made by Martin and Fuhrmann (1941) on the dog, where summated tissue respiration gives 79% of the resting metabolism. It is concluded, therefore, that resting metabolism can be accounted for by summated tissue respiration and minimal functional activity.

However, it is well-known that the respiration rates of tissues show considerable variation, depending on the medium used. In serum, or in media containing the relevant metabolites (Krebs, 1950), they are often two or three times the values obtained in saline. It appears, therefore, that it is hard to decide which values should be considered to be the "true" metabolic rates of tissues corresponding to those *in situ*, so that these values, multiplied by organ weight, would give the total respiration of the organ in question. If, instead of saline, the previously mentioned authors had used serum, they would probably have obtained not 66%, but more than 100% of basal metabolic rate as summated tissue respiration.

Kleiber (1941; Weymouth, Field and Kleiber, 1942) investigated tissue respiration of liver from rats, rabbits, sheep, horse and cow, and found that $\underset{\sim}{Q}_{O_2}$ decreases to the same extent (namely, the $-\frac{1}{4}$ power) as does the basal metabolic rate per unit weight, according to the $\frac{3}{4}$ power rule. He concludes (1941; p. 422), therefore, that "the factors which determine the metabolic level *in vivo* seem still to be present in the surviving tissue cut out of the organism." Similarly, Weymouth *et al.* (1944) studied the $\underset{\sim}{Q}_{O_2}$ of the midgut gland of individuals of different size in the kelp crab *Pugettia*. They also found a systematic decrease of the $\underset{\sim}{Q}_{O_2}$ values with increasing size, paralleling the decrease in weight-specific basal metabolic rates of the intact animals. Krebs (1950) compared the $\underset{\sim}{Q}_{O_2}$ values of 5 tissues of 9 mammalian species. He found that, in general, the $\underset{\sim}{Q}_{O_2}$ values of the larger species are somewhat lower than the homologous values of small species; but there is no parallel decrease in the different tissues, nor a consistent relation to the decrease of weight-specific basal metabolic rate with increasing size. The greatest decrease of $\underset{\sim}{Q}_{O_2}$ in species of larger size is found in the liver (comparable to Kleiber's results though with some exceptions); but in the other tissues the decrease in $\underset{\sim}{Q}_{O_2}$ is much smaller than the decrease in basal metabolism.

There seems to be, as yet, no systematic investigation on the intraspecific size dependence of the $\underset{\sim}{Q}_{O_2}$ of different tissues. The present work (*cf.* Bertalanffy and Pirozynski, 1951; Pirozynski and Bertalanffy, 1952) was started before Krebs' investigation came to our attention.

The preliminary report of our results (Bertalanffy and Pirozynski, 1951) has been followed by an interesting investigation of tissue respiration of kidney and liver in growing chicken (Crandall and Smith, 1952) which confirms the results and conclusions of the present work. Three groups of chickens (body weight 66, 178, and 2350 gm.) were investigated. Apart from an early minimum of total metabolism as well as of liver $\underset{\sim}{Q}_{O_2}$ after hatching, characteristic of chicken and not present in the rat, the relations found correspond to our results: No correlation between body size and $\underset{\sim}{Q}_{O_2}$ in the kidney, a slight decrease of $\underset{\sim}{Q}_{O_2}$ ($\alpha \sim 0.1$) with increasing body size in liver slices.

A remark seems to be appropriate as to the relative value of interspecific and intraspecific comparisons. The first has, of course, the advantage of allowing a much greater range of body sizes to be compared; further, adult animals are compared so that developmental differences cancel out. On the other hand, although the physiological differences between newborn and adults are great, the same is true for the anatomical, physiological, biochemical, ecological, etc. differences even between related species, not to speak of comparisons "from the mouse to the elephant." The startling fact is that in spite of this, simple quantitative relations in basal metabolism can be established, intraspecifically as well as interspecifically.

Material and Methods

In our experiments male and female albino rats (Wistar strain) were used, representing a continuous series from newborn animals of 9 gm. body weight to adults of 392 gm. The animals, except for the newborn, were kept for 24 hours in individual cages prior to the experiment. All rats were fed Purina Fox Chow and tap water *ad libitum,* before the actual experiment took place. The diet was restricted to tap water only for 12 to 18 hours (basal metabolism regime). All animals were killed by breaking the cervical vertebra.

The organs to be investigated were carefully removed immediately after death and placed in ice-cold saline. The pieces of whole organs were sliced by free hand according to the method introduced by Deutsch and Raper (1936). Tissues were sliced up to the thickness of 0.4 mm. except the diaphragm, which was wholly removed from the animal by means of sharp scissors or a razor blade, and cut into two or three pieces. The muscular parts were then carefully separated from the adjacent connective tissue and placed in the flasks. Before transferring the slices to the Warburg flasks, they were gently dried by touching with a piece of hard filter paper. The slices of organs from newborn rats were generally prepared in the same manner except the brain, which was cut in half and minced by means of a forceps before being placed in the flask. A few embryonic tissues were obtained from pregnant rats. The smaller embryos of 25–35 mgm. body weight were transferred into the flask after removing the fetal membranes; the larger specimens were sliced using the same technique as with the organs of adult animals.

The oxygen consumption was determined by the direct method of Warburg using the technique described by Umbreit *et al.* (1949). The oxygen uptake in Krebs-Ringer-phosphate solution of pH 7.4 was measured in pure oxygen with carbon dioxide absorbed by alkali-soaked filter paper in the center well. The flasks attached to the manometers were placed in a constant temperature bath of 37.0° C. (± 0.1) and shaken at the rate of 124 oscillations per minute. The equilibration period in the water bath was approximately 20 minutes. The standard experimental period was 60 minutes with readings taken every 10 minutes. The dry weight of the investigated tissue was determined on an analytical balance after drying the slices for at least two hours at 105–110° C.

Higher Q_{O_2} values can be obtained in other media. However, in phosphate Ringer we found the Q_{O_2} values to remain approximately constant during the experimental period, except in the case of brain (Fig. 1), while if glucose or other metabolites are added, the Q_{O_2} values often decrease. Since our study amounts to a comparison under standard conditions, it was preferable to use a medium where there is no decrease of Q_{O_2} during the experiment, and therefore no need to make extrapolations.

The order of the differences to be expected if the decrease of basal metabolic rate is based upon differences in tissue respiration can be estimated as follows. Basal metabolism is generally proportional to a power of the weight:

$$M = bW^{\alpha}, \tag{1}$$

where M is the rate of basal metabolism, W the body weight, the exponent α indicates the slope of the regression line in log-log plot, and b is a constant, indicating the extrapolated value of M for $W = 1$. The dependence of metabolism on body size is a special case of the general law of allometric growth (*cf.* Bertalanffy, 1951a). In the case of the surface rule, $\alpha = \frac{2}{3}$. The surface rule holds, although with qualifications, for intraspecific comparison in the rat (Bertalanffy, Mueller and Racine, unpublished); this is also stated by Kleiber (1947), according to Benedict's data (1938). Therefore,

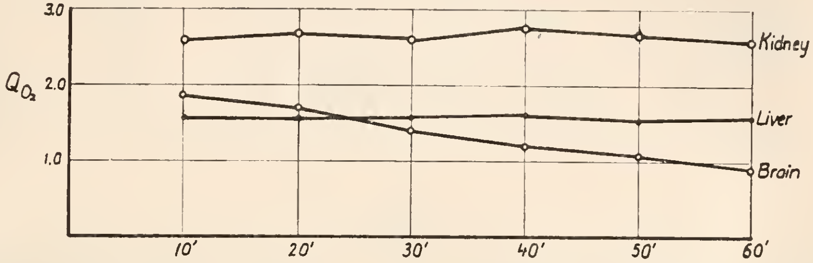

FIGURE 1. Q_{O_2} as a function of time. Typical experiments.

$$M/W = bW^{-1/3} \tag{2}$$

applies for metabolic rate per unit weight. Thus, the exponent α should be $-.33$ in the case of the surface rule (or $-.25$ for the ¾ power rule). If, for example, the weights compared are $1:2:4:8:16$, metabolic rates per unit weight (a measure of which is Q_{O_2}) should decrease in the ratio $1:.79:.63:.50:.4$, in the case of the surface rule. Differences of this order should be readily detected by the Warburg method.

The statistical evaluation of the data, and calculation of α, b, $S_{(\log y \cdot \log x)}$ (standard error), and ρ (coefficient of correlation) were made according to the method indicated by Brody (1945, pp. 398 ff.). In Figures 2–8, the central line gives the regression of Q_{O_2}, and the two parallel lines give the standard error in per cent, including ⅔ of the cases.

RESULTS

The results are indicated in Table I and Figures 2–8. As to the individual organs, the following remarks can be made.

TABLE I

Statistical evaluation of the relation of Q_{O_2} to body size in organs of the rat

Organ	N	α	b	$S_{(\log y \cdot \log x)}$	ρ
Heart	27	−0.050	10.83	0.102	0.229
Lungs	30	−0.085	10.33	0.073	0.501
Liver					
1st cycle:	30	−0.116	13.65	0.063	0.497
2nd cycle:	15	−0.018	8.59	0.074	0.107
Brain cortex	30	+0.047	6.89	0.064	0.263
Kidney cortex	43	+0.030	13.91	0.060	0.170
Thymus					
1st cycle:	11	−0.263	20.11	0.032	0.740
2nd cycle:	17	−0.253	29.46	0.068	0.514
Diaphragm	27	−0.258	18.92	0.066	0.929

Heart

The Q_{O_2} values are presented in Figure 2. There is a slight decrease with increasing body size, but the correlation coefficient is low, as can be seen from Table I.

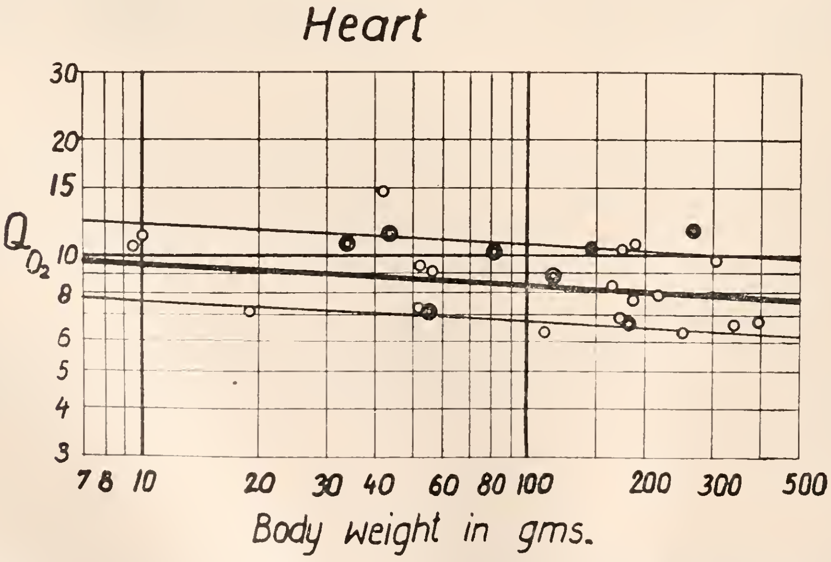

FIGURE 2. Q_{O_2} of heart in relation to body size. o = males, ◎ = females in Figures 2–8.

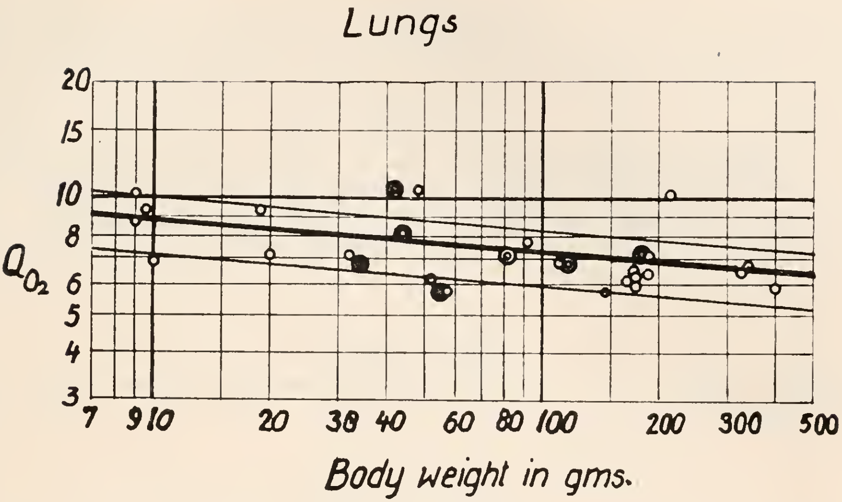

FIGURE 3. Qo_2 of lung in relation to body size.

Lung

The situation for the Qo_2 of lungs is similar to that of heart, as shown in Figure 3.

Liver

It appears that a break can be assumed in the allometric line of the Qo_2, somewhere in the region of 100 gms. body weight (Fig. 4). This break would correspond to a break in the curve of the relative growth of this organ (Bertalanffy and Pirozynski, 1952). It seems that at this body weight and in this time which

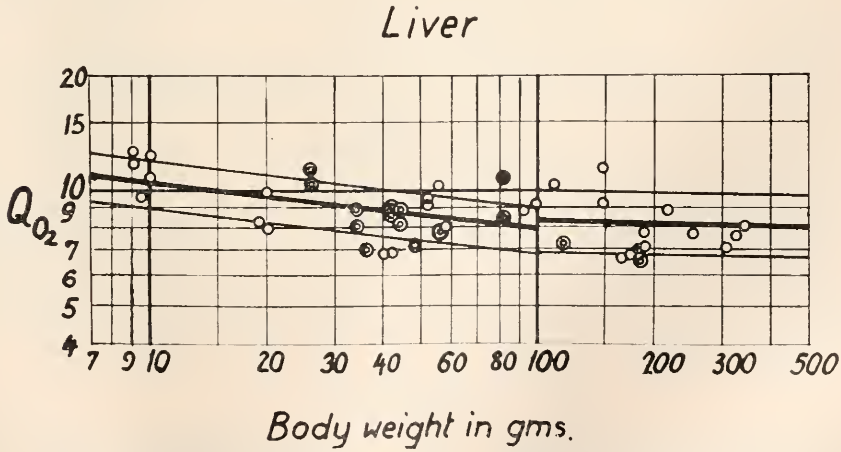

FIGURE 4. Qo_2 of liver in relation to body size.

corresponds to the start of puberty, a deep-reaching physiological change takes place which can be observed in different ways. According to Bertalanffy (1938, 1951a), the curve of total growth shows a break in this region, so that here the transition from the "first" to the "second growth cycle" occurs. Basal metabolism also undergoes a deviation here (Bertalanffy, Müller and Racine, unpublished data). Further, as already mentioned, there is a break in the relative growth and in the regression line of Q_{O_2} of the liver. These changes in total growth, in basal metabolism, in the relative growth of the liver, and in the tissue respiration of the liver are observed independently of each other which is a strong indication that they are different expressions of an actual change.

Kidney Cortex

The Q_{O_2} values (Fig. 5) remain practically constant over the range of body sizes investigated.

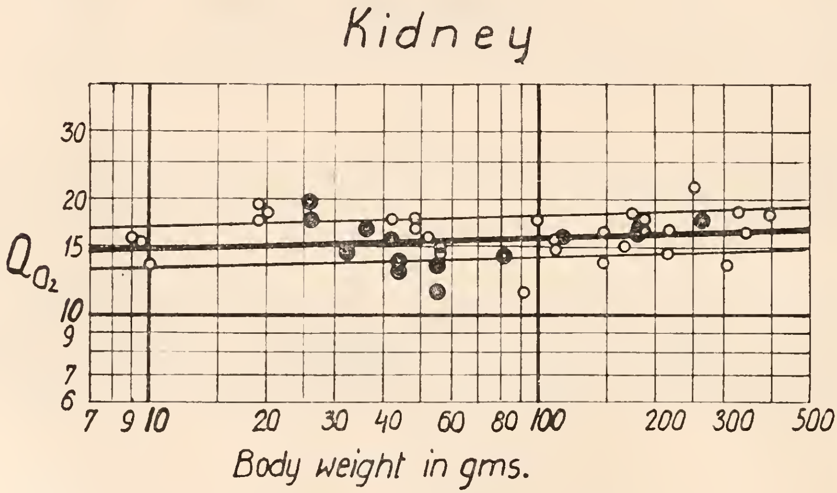

FIGURE 5. Q_{O_2} of kidney cortex in relation to body size.

Brain Cortex

There seems to be a slight increase of the Q_{O_2} of the brain with increasing body size (Fig. 6). According to Elliott (1948), the values for the respiration rate of brain show no obvious correlation with the size of the animals. Although our values for brain are lower than those reported by Elliott and others, because no glucose was used in our experiments, a slight upward trend is noticeable in Elliott's values for the rat, and this seems to correspond with our findings.

Diaphragm

The diaphragm is the only organ used in our experiments which shows a definite correlation of Q_{O_2} with body size, giving an exponent $\alpha = -.26$, and a high

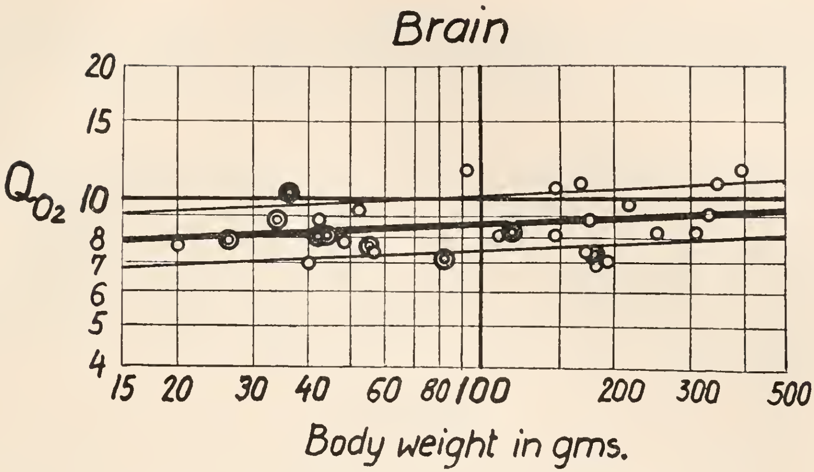

FIGURE 6. Q_{O_2} of brain cortex in relation to body size.

correlation coefficient, $\rho = .93$ (Fig. 7). It is to be noted that the diaphragm was not sliced as were the other tissues.

The marked decline of Q_{O_2} of the diaphragm with increasing body size may be connected with the continuous activity of this organ in respiration, since it serves oxygen uptake which, in total metabolic rate, shows a similar dependence on body weight. Also in interspecific comparison of adult rats and mice, there is a similar correlation of the Q_{O_2} of diaphragm to body size as is found intraspecifically

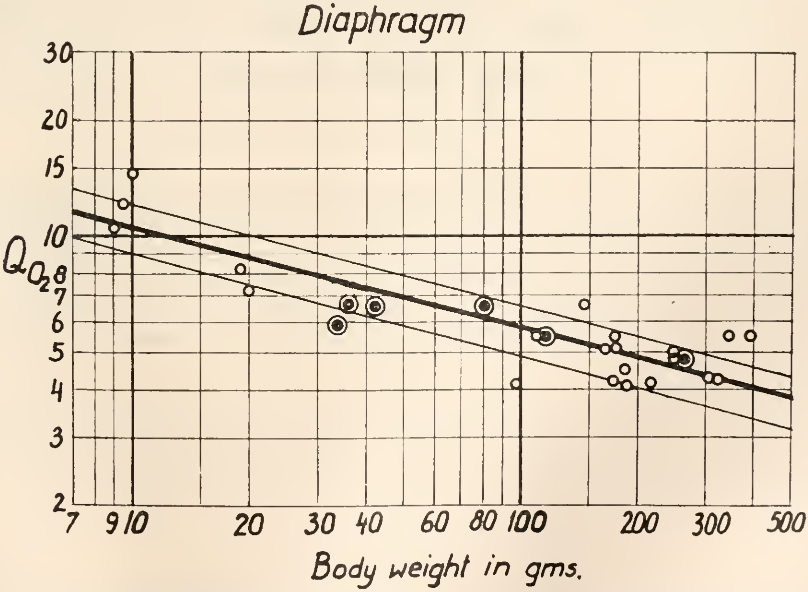

FIGURE 7. Q_{O_2} of diaphragm in relation to body size.

(Bertalanffy and Estwick, 1953). The size dependence of Q_{O_2} of the diaphragm may be connected with the fact that respiratory rate is higher in small as compared to larger animals. This will be studied in further experiments.

Thymus

In the Q_{O_2} values for thymus, a periodization (Fig. 8) can be found since they can be divided into two regression lines, each with a slope of approximately $-\frac{1}{4}$, and interrupted at about 100 gms. body weight. The break would again correspond to the "critical period" mentioned above, as well as to the involution of the thymus which, measured as relative growth, manifests itself as a break in the allometric plot of weight of thymus against body weight (Bertalanffy and Pirozynski, 1952).

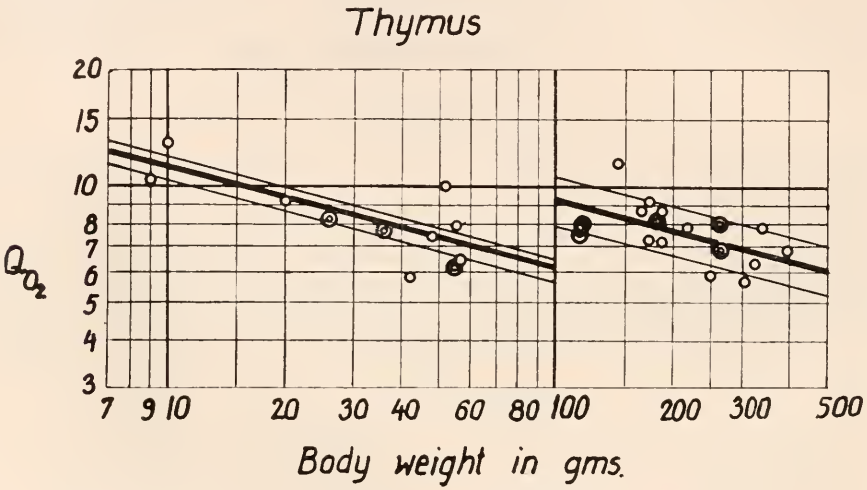

FIGURE 8. Q_{O_2} of thymus in relation to body size.

In conclusion, it can be stated that our experiments do not show systematic differences of Q_{O_2} accounting for the decrease of weight-specific total metabolic rate with increasing body size. A slight decrease may be noted in liver, lung and heart, but is definitely smaller than expected from the surface or $\frac{3}{4}$ power rule, and the correlation with body size is low. A slight increase in Q_{O_2} with increasing size may be noted in the brain. The diaphragm shows a definite correlation of Q_{O_2} to body size.

It is further to be noted that the logarithmic Q_{O_2} plots of two organs, namely, liver and thymus, show a break, both in the region of about 100 gm. These two organs are also the only ones among those investigated which show a break in their relative growth at the same period (Bertalanffy and Pirozynski, 1952).

Tissue respiration in embryos

A small number of determinations were made with embryos and embryonic tissues. Our values for naked fetuses are given in Table II. These results con-

TABLE II

Q_{O_2} of rat fetuses of different size

Body weight in mgm.	24.1	27.2	33.0	650.0
Q_{O_2}	−6.24	−7.38	−7.82	−6.12

firm those of Kleiber, Cole and Smith (1943) who give for rat fetuses (naked) a mean Q_{O_2} of − 7.2, with which our values are in good agreement. These authors estimate that the rate of oxygen consumption of the 13-day old rat fetus *in vitro* is only $\frac{1}{10}$ of the rate to be expected if the fetus behaved metabolically like a small independent homeotherm according to the $\frac{3}{4}$-power rule, or $\frac{1}{20}$ of the rate to be expected according to the surface rule. The fetal metabolic rate per unit moist weight is of the same order of magnitude as that of adult rats and considerably smaller than that of newly born to 12-day old rats. A few determinations of fetal organs are given in Table III. It appears that the Q_{O_2} of fetal liver is characteristically smaller than that of newborns.

DISCUSSION

1. *Interspecific and intraspecific comparison*

In order to compare intraspecific and interspecific size dependence of tissue metabolism, the data of Krebs (1950) were given a statistical treatment identical to that used for our own experiments. Krebs gives the Q_{O_2} for lung, liver, kidney cortex, brain cortex, and spleen in 9 species of mammals, ranging from the mouse to the horse, as found in the improved media introduced in his paper. The mean values of Q_{O_2}, as given by Krebs (p. 259, Table IV) were calculated. A comparison of Tables IV and I shows the following facts.

TABLE III

Q_{O_2} of some fetal organs

Weight of fetus in grams	Brain cortex	Kidney cortex	Liver
0.65	−6.99		−7.45
	−7.52		−9.18
			−8.8
2.9	−8.08	−15.24	−7.13
			−9.38
			−8.83
			−8.14

1) The decrease in Q_{O_2}, as measured by the exponent α, is, in all tissues studied by Krebs, considerably smaller than would correspond to the ¾-power rule (in the order of $\alpha = -.06$ to $-.14$, as compared to $-.25$, demanded by this rule). Therefore, interspecific comparison shows in the same way as does intraspecific comparison, that variations in tissue respiration, measured by the Warburg technique, cannot be the decisive factor for the decrease of weight-specific basal metabolic rate. Insofar as the brain is concerned, Krebs' determinations correspond well with those given by Elliott (1948). Elliott gives respiration per unit fresh weight in mice, rats, guinea pigs, rabbits, dogs, man and beef. He finds that respiration of cortex slices decreases inversely with body size, the exponent α being $-.1$. Since Elliott's regression line is drawn by estimate and without statistical calculation, this value is practically identical with the value obtained by statistical evaluation of Krebs' data ($-.07$).

2) The interspecific and intraspecific α-values are partly similar (liver, lung), partly they are significantly different (brain, kidney). This is an expression of a fact that is found also in morphological phenomena (Bertalanffy and Pirozynski, 1952), namely, that intraspecific and interspecific allometry (a special case of which are the relations between metabolic phenomena and body size) need not necessarily be identical.

TABLE IV

Statistical evaluation of Krebs' data on the relation of Q_{O_2} to body size in nine mammalian species

Organ	α	b	$S_{(\log y \cdot \log x)}$	ρ
Brain cortex	−0.069	35.69	0.029	0.969
Kidney cortex	−0.064	43.79	0.057	0.882
Liver	−0.115	23.43	0.063	0.949
Spleen	−0.139	20.21	0.059	0.968
Lung	−0.095	11.18	0.098	0.846

3) The values of the integration constant b, which indicates the extrapolated value of Q_{O_2} for a weight $W = 1$ gm., are much higher in Krebs' than in our data. This is a consequence of the fact that the media applied by Krebs give higher absolute Q_{O_2} values than those obtained in Ringer-phosphate, as used in our experiments.

4) The values of the standard errors $S_{(\log y \cdot \log x)}$, as given for our own and Krebs' data, are not directly comparable. Table I gives the standard errors, calculated for our individual determinations, while Table IV is calculated, as a matter of convenience, for the mean values obtained from a larger number of determinations. Nevertheless, the comparison is rather favorable for our results because the standard errors for our individual determinations are not larger than those for the average values given by Krebs.

5) The correlation between body size and tissue metabolism, as expressed by ρ, is much higher in interspecific than in intraspecific comparison. In other words, the factor of body size plays a greater role in determining the rate of tissue metabolism within different species of different size, than it does in determining tissue metabolism of individuals of different size within the same species.

2. *The explanation of the relation between metabolism and body size*

Our results lead to a number of conclusions with respect to possible explanations of the rule that weight-specific metabolic rate decreases with increasing body size.

1) A first explanation is that this decrease, as expressed in the surface or ¾-power rule, is due to intracellular factors, *i.e.,* to a corresponding decrease of the Q_{O_2} of the tissues the sum of which is the organism. Krüger (1940) suggested that the surface rule is based upon the principle of chemical allometry (Needham, 1934), and possibly upon the decrease in concentration of respiratory enzymes with increasing body size. Such decrease is actually found with respect to some systems taking part in respiration. According to Rosenthal and Drabkin (1943), the cytochrome *c* concentration decreases in the series mouse, rat, rabbit, dog, pig, man, and horse, with the −.278 power of body weight, *i.e.,* approximately according to the ¾-power rule. A decrease in glutathione with increasing body size was found by Gregory and Goss (1933) and Patrušev (1937). A similar concept was advanced by Weymouth *et al.* (1944). The coincidence of the values for total and liver respiration in rats, rabbits, and sheep, and equally for total respiration (interspecific and intraspecific comparison) and respiration of the midgut gland in crustaceans, leads these authors to the conclusion that "the regressions of the weight-specific rates for the different tissues apparently form, in the log-log plot, a family of parallel lines, some high and some lower, corresponding to the intensity of respiration, but all showing the same slope as the regression of the weight-specific rate of the entire animal" (p. 68).

There are two objections to be made against this argument. As far as crustaceans are concerned, total metabolism in *Pugettia* follows, according to Weymouth *et al.* (1944), the ¾-power rule. Investigations with other crustaceans (isopods: Müller, 1943b; Bertalanffy, 1951b; *Daphnia:* Jančařík, 1948; *Artemia:* Bertalanffy and Krywienczyk, 1953) show, however, that here the surface rule applies.

With respect to the general viewpoint, our results show that the extrapolation to all organs from one organ investigated—the liver in the rat, the midgut gland in crabs—is not justified. Our results, obtained in intraspecific comparison, correspond in this respect with the interspecific comparison, as carried through by Krebs: The regressions of Q_{O_2} with respect to body size are different for the various organs.

2) Krebs (1950), not finding a systematic decrease of the Q_{O_2} values investigated which would correspond to the decrease of weight-specific total metabolic rate, concludes (p. 266) that "the characteristic differences in the basal rate of heat production in animals of different size are to be attributed mainly to variations in the Q_{O_2} of the musculature." Krebs did not investigate the Q_{O_2} of muscles in animals of different size. His conclusion is based upon the fact that the muscles play a leading part in thermoregulation which, according to the classical explanation given by Rubner, is at the basis of the surface law. Krebs offers this explanation for interspecific comparison of metabolic rates in the series of mammals, but naturally it could be applied also to intraspecific comparison.

Two viewpoints are to be distinguished in this hypothesis. *a*) Insofar as muscular activity is a means of thermoregulation, this cannot be the basic principle in the decline of weight-specific metabolic rate with increasing body size. For the latter is a phenomenon in no way specific of, or limited to homeotherms, but universal in most animal phyla (Bertalanffy, 1951b). The surface rule applies, even more unequivocally than in mammals, to cold-blooded vertebrates and many invertebrate classes. It was found to apply in roundworms (*Ascaris:* Krüger, 1940); in certain molluscs (*Lamellibranchiata:* Weinland, 1919; Ludwig and Krywienczyk, 1950; *Prosobranchia:* Krywienczyk, 1952a); in crustaceans (*Branchiopoda:* Jančařik, 1948; Bertalanffy and Krywienczyk, 1953; *Isopoda:* Müller, 1943b; Will, 1952); in fish (Bertalanffy and Müller, 1943) and reptils (Kramer, 1934). A decline of metabolic rates corresponding to the ¾-power rule is found in turbellarians (Bertalanffy and Müller, 1943) and in pond snails (*Limnaeidae:* Bertalanffy and Müller, 1943; Füsser and Krüger, 1951; Krywienczyk, 1952b). The only groups where no decline of weight-specific metabolic rates is found, *i.e.,* where total metabolism is directly proportional to weight, are land snails (*Helicidae:* Liebsch, 1929; Bertalanffy and Müller, 1943) and insects (Kittel, 1941; Bertalanffy and Müller, 1943; Müller, 1943a; Will, 1952)—possible due to pecularities of their respiratory mechanisms. Thus, homeothermy and muscular activity in its service is certainly not the basic principle in the size dependence of metabolic rate.

In mammals, the condition of thermoneutrality of environment, as applied in basal metabolism determination, amounts to minimize the energy expense for thermoregulation. The environmental temperature is so adjusted as to keep the body temperature normal without regulation, the heat arising as a by-product of the reactions in metabolism being sufficient to maintain body temperature. Only in conditions of non-thermoneutrality, muscular activity in the form of increased tension, shivering, etc. comes into play. This represents an excess superimposed over basal metabolic rate which is considerable indeed even in rather slight deviations from thermoneutrality, and which is measurable in appropriate experiments. This, however, does not concern basal respiration. Considering the fact that the decline of metabolic rate with increasing body size is a general phenomenon found also in animals without thermoregulation, it may be safely concluded that variations in the $\mathrm{Q}_{\mathrm{O}_2}$ of musculature due to thermoregulation are not the factor responsible for the phenomenon in question.

b) It remains to be seen whether variations in the tissue respiration of skeletal muscle can account for the decline of weight-specific metabolic rate. Assuming a decrease of $\mathrm{Q}_{\mathrm{O}_2}$ of musculature according to the surface or ¾-power rule, and considering the percentage of musculature in relation to total weight in small and large rats, according to Donaldson (1924, Table 124, p. 184), a simple estimate shows that the decline in muscular $\mathrm{Q}_{\mathrm{O}_2}$ would not be sufficient to account for the decline in weight-specific basal metabolic rate of the entire animal.

The relation of $\mathrm{Q}_{\mathrm{O}_2}$ of skeletal muscle to body size was recently studied by Bertalanffy and Estwick (1953). The $\mathrm{Q}_{\mathrm{O}_2}$ of leg muscles of rats decreases, with increasing body size, with $\alpha = -.07$, *i.e.,* much less than would correspond to the surface or ¾-power rule. Interspecifically, the $\mathrm{Q}_{\mathrm{O}_2}$ of skeletal muscle of adult mice (25–30 gm.) is similar to that of adult rats (over 300 gm.).

Thus there seems to be no indication that the decrease of weight-specific metabolic rate is to be explained by variations of the $\mathrm{Q}_{\mathrm{O}_2}$ of musculature.

3) A third hypothesis often offered is that the mass of "metabolically active" organs decreases relatively with increasing body size. The high metabolism of small and young animals, as compared to the adult animals, would be based on the

fact that the inner organs which have a high oxygen consumption, are comparatively larger in the first (Kestner, 1934; Blank, 1934). Blank comes to the conclusion that the relatively larger size especially of the heart, kidney, intestinal tract and nervous system in smaller animals is responsible for their high oxygen consumption. However, already in 1942 (Bertalanffy, p. 198 ff.; 1951a, p. 249 ff.) we had shown by an analysis of the relative growth of the organs concerned that the latter is much too involved and too varying from one organ to the other, intraspecifically as well as interspecifically, that its combined effect is likely to result in a phenomenon so universal as the systematic decrease of weight-specific metabolic rate according to the simple power formula (on relative growth of inner organs of the rat; *cf.* Bertalanffy and Pirozynski, 1952).

A quantitative estimate may show that this factor cannot be the decisive one in the relative decrease in basal metabolism. As mentioned above, Field *et al.* (1939) have calculated summated tissue respiration for the mature rat. We have made a similar estimate for the 10-gm. rat, using Field's values for oxygen consumption per unit fresh weight for the individual organs, the wet weights of organs according to Donaldson (1924), and assuming a regression for Qo_2 of skeletal muscle, liver, lung, and heart as found in the present experiments. As calculated by Field *et al.*, summated tissue respiration gives 110 cc. O_2/hr. for the 150-gm. rat. The calculation carried through as mentioned above, gives a summated tissue respiration of *ca.* 8.7 cc. O_2/hr. for the 10-gm. rat. Since the basal metabolic rate of an animal of 150 gm. is *ca.* 165 cc. O_2/hr., that of an animal of 10 gm. *ca.* 25 cc. O_2/hr., summated tissue respiration as based upon Qo_2-measurements in saline, accounts for 66% of basal metabolism in the mature rat, but only for 35% in the 10-gm. rat. Although such estimate is, of course, crude and over-simplified, it shows that *a*) the different proportion of inner organs in small and large animals does not account for the differences in their basal metabolic rates; and *b*) that respiration in a young animal must be considerably higher than the values obtained in saline.

4) Another possible correlation is that between tissue respiration and age. According to Hawkins (1928) who studied liver slices of three groups of rats, aged 3–21 days, one year, and 22 months, respectively, and Pearce (1936) who used liver, heart and kidney of two groups (4–9 and 50–60 weeks) of mice, Qo_2 values decrease with age. Although our experiments show a decrease of Qo_2 with increasing body size in some organs, they do not show a definite relation between Qo_2 and age. Our determinations are not in contradiction with those of the authors mentioned. However, one would expect that the organs which show the most conspicuous histological changes (deposits of lipofuscins) and are particularly involved in the process of aging, namely, brain and heart, should also show definite changes in respiration with increasing age. This is not the case.

5) Our experiments seem, therefore, to contradict explanations of the decline in basal metabolic rate with increasing size which are based upon factors lying in the tissues themselves, and active, therefore, also in tissue respiration as observed with the Warburg technique. It appears that the decline in basal metabolic rate depends upon regulative factors lying in the organism as a whole. Many such factors can be taken into account: hormonal and neural regulators, supply of oxygen and metabolites especially of the Krebs cycle, etc. Variations in the energy expense for minimal functional activity (heart, lungs, kidney, etc.) are naturally also to be taken into account.

Our conclusion that respiration as determined *in vitro* does not necessarily correspond to respiration *in situ*, is derived from a study of normal animals and tissues. It may be mentioned that similar conclusions result from the study of malignant tissues. Potter (1951), investigating the Krebs cycle in tumors, states (p. 569) that "experiments with whole animals using fluoroacetate to block citrate oxidation *in situ* suggest that the enzyme activity of tissues *in situ* may be different than either the homogenate or slice would indicate." It may be hoped, therefore, that further study of the factors modifying tissue respiration can throw light on the problem of regulation of metabolism under conditions of normal as well as pathological growth.

Taking, as a starting point, the metabolic level in a young animal which perhaps roughly corresponds to the metabolic rate as observed in media containing all necessary metabolites, such as serum or the solutions indicated by Krebs (1950), it would seem that tissue respiration is damped in a larger animal, to a level roughly corresponding to that obtained in saline. If this is the case, it is suggestive to remark that also the growth of organs is limited by organismic factors. The "growth potency" of tissues is not limited by factors lying in those tissues themselves. This is shown by the fact that tissues which would not grow within the organism, do so if removed from the organism and cultivated *in vitro;* similarly, if the steady state and the "balance of organs" are disturbed, as in regeneration and compensatory hypertrophy; and finally under pathological conditions, in malignant growth. It may be that the metabolic activity of organs is bridled, as it were, within the organism, so that the systemic decline of weight-specific metabolic rate with increasing size takes place; that the same is true for their growth potency; and that both factors are connected. Investigation of these factors may perhaps lead into a deeper insight on growth, normal as well as malignant.

SUMMARY

1. The relation between tissue respiration and body size was investigated in the rat. Determinations of Q_{O_2} were made on heart, lung, liver, kidney cortex, brain cortex, diaphragm, and thymus of animals ranging from 9 gm. to 392 gm. body weight, including some determinations on fetuses and fetal tissues. A statistical evaluation of *ca.* 230 experiments is given.

2. The diaphragm is the only organ investigated to show a definite and significant correlation between rate of tissue respiration and body size. Liver and thymus show a break in the regression line which corresponds to a number of other characteristic changes in metabolism and growth.

3. The experiments do not show systemic differences in tissue respiration accounting for the decrease of total metabolic rate with increasing body size.

4. A comparison between intraspecific and interspecific size-dependence of tissue metabolism is made.

5. The current theories on the systematic decrease of weight-specific metabolic rate, as expressed in the surface or ¾ power rule, are discussed in the light of the experiments presented. It is shown that none of the explanations proposed (decline of total metabolic rate as based upon decrease of the rate of tissue respiration, upon thermoregulation, upon decrease of Q_{O_2} of musculature, upon the relative decrease of "metabolically active" organs, upon age) in consistent. It appears

that the decline in basal metabolic rate depends on regulative factors lying in the organism as a whole.

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SUMMATION AND INHIBITION FOLLOWING CONTRALATERAL STIMULATION OF THE TARSAL CHEMORECEPTORS OF THE BLOWFLY

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In one of the efforts to arrive by behavioral methods at an understanding of the fundamental process of stimulation of tarsal chemoreceptors by unacceptable compounds it was discovered that the action of opposing stimuli, as for example sugar and alcohol, could be either ipsilateral or contralateral (Dethier, 1950). Normal proboscis extension in response to sugar could be prevented by adding propanol to sucrose solutions or by stimulating one leg with propanol alone while the opposite leg was exposed to sucrose. The fact that prevention of proboscis response could result from interaction in the central nervous system of opposing patterns of activity from opposite legs directed attention to the existence of central inhibtion in this insect.

In addition to providing information relative to the interaction of *unlike* stimuli, the early experiments yielded data which pointed to the existence of central summation following stimulation by *like* stimuli. However, another series of experiments in which contralateral stimulation by like stimuli was investigated for the olfactory sense (Dethier, 1952a) suggested that the observed results might be simply an expression of a statistical bias introduced by doubling the available number of receptors. When rejection thresholds for pentanol vapor were compared in flies with a single antenna and those with both antennae, it was observed that thresholds were lower for bilateral stimulation than for unilateral; but the decrease of bilateral over unilateral was never greater than could be satisfactorily accounted for on a simple probability basis (*cf.* Smith and Licklider, 1949). Thus, while these tests did not negate the existence of true neural contralateral summation, they did not necessarily affirm it. Since the experiments were designated to measure the per cent response at only one or two test concentrations rather than to ascertain values for complete threshold frequency distribution, a rigorous statistical analysis was not possible. Nor did it appear feasible, because of the difficulty of controlling vapor concentrations, to attempt to resolve the problem with the antennal preparation. Tarsal chemoreceptors are clearly more amenable to complete analysis. Consequently, the investigation described herein was undertaken to extend earlier tarsal as well as olfactory data in an effort to permit a clearer statement of the status of contralateral summation and inhibition in the response of the blowfly to chemicals.

METHODS

The solution of two immediate problems was undertaken: (1) to determine whether or not simultaneous stimulation of opposite legs by like stimuli sums in the central nervous system; (2) to determine to what extent unlike stimuli inhibit each other. Experimental methods fell into two categories. In the first, tests were conducted with one-legged and two-legged flies. These insects were prepared as follows. One- to two-day old flies (*Phormia regina*) from a stock culture were anesthetized with carbon dioxide and attached by the wings to wax-tipped applicator sticks. In the case of one half of the flies, all legs except one prothoracic were fastened into the wax. Of this group of flies, one half had the right prothoracic leg free and the other half, the left. In the remaining group of flies all legs except the prothoracic pair were immobilized. Measurements were then made of the responses of one-legged and two-legged flies to sucrose, propanol in water, propanol in 0.1 M sucrose, and HCl in water.

In the second series of experiments the responses of two-legged flies were tested in partitioned dishes. The vessels used were Stender dishes 45 mm. in diameter with a cover glass 40 mm. long fastened on edge across the middle with paraffin. The top of the cover glass was set flush with the top edge of the dish. A different solution could now be poured into each half of the dish, care being taken to insure that the liquids rose only to such a level as to make concave rather than convex menisci at the partition.

With this device it was possible to stimulate opposite prothoracic legs of a fly simultaneously with different solutions by causing him to straddle the partition. In practice, execution of a test required the utmost delicacy and the patience of Job. A captive fly suspended in air had a tendency to keep its legs crossed or flexed at the tibio-tarsal joint. A proper test cannot be made until both legs are extended and spread apart. When a fly had assumed this position, it was lowered gently over the partition. Care had to be exercised that both legs touched the respective solutions at approximately the same moment and that the partition was not grasped by either leg. Under favorable conditions, proboscis extension, if adequate stimulation was supplied, occurred in the usual manner.

This technique was employed to test unilateral versus bilateral stimulation by a single compound and to investigate the effects of opposing stimuli acting simultaneously on opposite legs. In the first instance, unilateral stimulation, by sucrose, for example, was accomplished by forcing the fly to straddle a partition which separated a sucrose solution from pure water. For bilateral stimulation sucrose was placed on both sides of the partition. Similarly, the stimulating effects of various combinations of sucrose, water, HCl, NaCl and propanol were investigated. The concentrations of HCl and propanol chosen for stimulation were those which, under normal conditions of testing, would yield responses well above the 50% level. The concentration chosen for NaCl was nearer to the 50% level. The number of flies used in each experiment varied from 60 to 800.

In every case, except where noted otherwise, the flies were random sampled, *i.e.,* a different group was tested at each concentration in the series, and the per cent which responded was noted. Median threshold values were calculatd according to the method of Bliss (1938) (*cf.* also Dethier and Chadwick, 1948).

RESULTS

Unilateral versus bilateral stimulation by sucrose

Originally a straightforward comparison of the sucrose thresholds of one-legged and two-legged flies was made. In these experiments the flies were tested in ascending order; that is, each fly was exposed successively to each concentration of the test solution, beginning with a subliminal concentration, until one was reached which elicited a proboscis extension. Since there was a real possibility that one-legged and two-legged flies were not strictly comparable, *i.e.*, that the number of legs immobilized might affect the general behavior of the fly, additional tests were run with two-legged flies, one of whose legs was tethered with a fine silken thread. Each such fly was tested with the tethered leg suspended above the solution (unilateral stimulation) and then with both legs in contact with the solution (bilateral stimulation). Finally the partitioned dish, as already described, was employed. This time the experiments were run in random rather than ascending series. As an added precaution and to insure accuracy of results most of the experiments with partitioned dishes were coded so that the experimenter was unaware of the contents of the dishes.

Regardless of the method of testing employed the results were similar (Table I). The unilateral threshold was always significantly higher than the bilateral threshold (Fig. 1). In these experiments, the absolute values obtained from random testing are not directly comparable with the values obtained from ascending testing because it was not possible to use flies of uniform age and nutritional background throughout the entire study. When these factors are controlled, ascending thresholds are higher than random thresholds (*cf.* Dethier, 1952b).

Contralateral stimulation by unlike compounds

In order first to survey the situation with regard to such representative unacceptable compounds as NaCl, HCl, and propanol, spot tests were undertaken

TABLE I

Comparison of bilateral and unilateral thresholds of response of Phormia regina to sucrose, HCl, and propanol†

Compound and technique of testing‡	Conc. at which 50% of flies respond	Log median ±2.575 S. E.	a + S. E.	b + S. E.	$\bar{x}$	No. of insects tested
Sucrose, one leg, ascending	0.0037	−2.433 ±0.191	5.140 ±0.050	0.713 ±0.082	−2.237	173
Sucrose, two legs, ascending	0.0018	−2.749 ±0.149	5.387 ±0.055	1.163 ±0.122	−2.416	173
Sucrose/water (=1 leg)	0.1010	−0.996 ±0.515	4.326 ±0.078	0.640 ±0.105	−1.993	360
Sucrose/sucrose (=2 legs)	0.0200	−1.699 ±0.258	4.672 ±0.021	0.812 ±0.030	−2.103	360
*Sucrose/water (=1 leg)	0.0177	−1.930 ±0.258	4.900 ±0.045	0.474 ±0.067	−2.141	800
*Sucrose/sucrose (=2 legs)	0.0021	−2.670 ±0.149	5.308 ±0.049	0.991 ±0.096	−2.359	800
Propanol in water, 1 leg	0.607	−0.217 ±0.156	5.037 ±0.073	1.206 ±0.137	−0.186	360
Propanol in water, 2 legs	1.05	0.020 ±0.162	4.734 ±0.068	1.160 ±0.121	−0.209	420
Propanol/water (=1 leg)	1.34	0.128 −0.170	5.011 −0.182	2.770 −0.589	0.132	60
Propanol in 0.1 M sucrose						
1 leg	0.59	−0.232 ±0.098	5.115 ±0.107	2.867 ±0.369	−0.192	200
2 legs	0.95	−0.021 ±0.098	4.832 ±0.105	2.770 ±0.351	−0.082	200
Propanol/0.1 M sucrose, random	1.23	0.088 ±0.165	5.077 ±0.083	1.332 ±0.253	0.146	240
Propanol/0.1 M sucrose, ascending	1.076	0.032 ±0.155	5.132 ±0.134	2.254 ±0.311	0.090	120
HCl in 0.1 M sucrose, 1 leg	1.041	−1.391 ±0.163	4.877 ±0.068	1.107 ±0.147	−1.502	70
HCl in 0.1 M sucrose, 2 legs	0.051	−1.298 ±0.109	4.773 ±0.069	1.441 ±0.142	−1.456	70

* As a check against the method all solutions in these series were coded.

† The 4th, 5th, and 6th columns give the calculated values for a, b, and $\bar{x}$ in the equation $Y = a + b(X - \bar{x})$, which is the regression of per cent flies responding, Y, expressed as probits on log concentration, X.

‡ Unless stated otherwise all tests represent random sampling. The slanting line symbolizes a divided dish. For explanation of different sucrose thresholds see text.

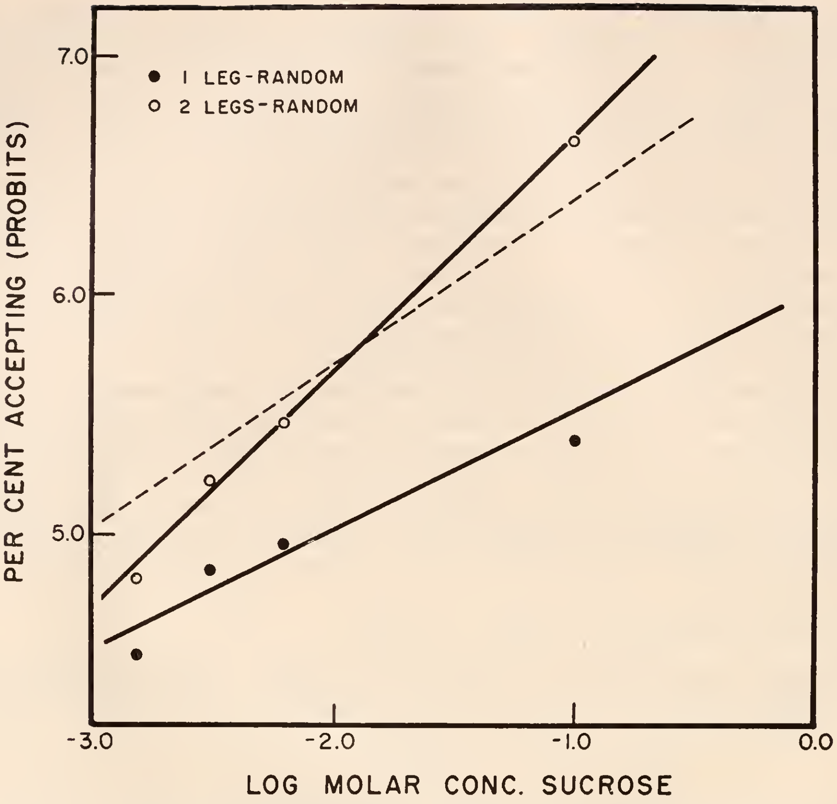

FIGURE 1. Comparison of the distribution of acceptance thresholds for sucrose, as a function of concentration, for flies stimulated unilaterally or bilaterally. The broken line represents the theoretical distribution of bilateral thresholds (two-legged flies) calculated from the expression $1-q^2$ where q equals the fraction of the population of one-legged flies not responding.

by means of a series of partitioned dishes. As had been done earlier in the olfactory studies (Dethier, 1952a) the procedure was adopted of selecting a single concentration at which measurements were made of the change in per cent of the population responding as a different number of appendages was stimulated. This course of action was followed for the sake of simplicity in lieu of varying the concentration to ascertain what strength of stimulus was required to elicit 50% response (median threshold) under each changed experimental condition. However, the quantitative information yielded by such spot testing cannot be utilized to its fullest extent in the absence of additional experiments because there is no way of knowing without further testing what relation the slopes of the threshold distribution curves of one-legged and two-legged flies bear to each other. It will be shown further on that the results of spot tests are substantiated by data from more detailed experiments.

The results obtained with the various spot combinations are arranged in series of pairs (Table II) for purposes of comparison. The following numbers correspond to the comparison numbers in the table, and the nature of the comparison is further indicated here by the symbols in parentheses. A slanting line indicates the partition dividing the two solutions, s signifies sucrose, and P signifies a repellent compound.

I (H_2O/s vs. P/s). All three unacceptable compounds can prevent a response to sucrose even when acting on the leg opposite to that leg which is being stimulated by sucrose. This is shown by the increase in per cent rejection over that recorded when water alone is paired with sucrose.

Table II

Comparison of mean rejection under different conditions of contralateral stimulation

Comparison no.	2 M propanol vs. 0.1 M sucrose and H_2O		1 M NaCl vs. 0.1 M sucrose and H_2O		0.1 M HCl vs. 0.1 M sucrose and H_2O	
	Condition of test	Mean % rejection	Condition of test	Mean % rejection	Condition of test	Mean % rejection
I	H_2O/s	47.2	H_2O/s	58.7*	H_2O/s	46.6
	P/s	70.9	$NaCl/s$	60	HCl/s	65.3
II	$H_2O/P+s$	87.2	$H_2O/NaCl+s$	65.6*	$H_2O/HCl+s$	91.3
	$s/P+s$	54.5	$s/NaCl+s$	53	$s/HCl+s$	73.3
III	$s/P+s$	54.5	$s/NaCl+s$	53*	$s/HCl+s$	73.3
	$P/P+s$	94.5	$NaCl/NaCl+s$	66	$HCl/HCl+s$	99
IV	$H_2O/P+s$	87.2	$H_2O/NaCl+s$	65.6*	$H_2O/HCl+s$	91.3
	P/s	70.9	$NaCl/s$	60	HCl/s	65.3
V	P/s	70.9	$NaCl/s$	60*	HCl/s	65.3
	$P/s+P$	94.5	$NaCl/s+NaCl$	66	$HCl/s+HCl$	99
VI	$P+s/P$	94.5‡	$NaCl+s/NaCl$	66*	$HCl+s/HCl$	99†
	$P+s/H_2O$	87.2	$NaCl+s/H_2O$	65.6	$HCl+s/H_2O$	91.3
n	110		160		150	

* There is no significant difference between the means compared in these pairs.

† Difference is significant at $P = 0.02$.

‡ Difference is significant at $P = 0.05$. All other differences are highly significant.

n = number of flies tested in each paired comparison.

II ($H_2O/P + s$ vs. $S/P + s$). An appreciably lower rejection is obtained with sucrose on both sides of the partition and a repellent compound on one side only. This result is in agreement with the fact that bilateral stimulation by sucrose is followed by greater acceptance than is unilateral stimulation.

III ($s/P + s$ vs. $P/P + s$). This comparison seems to show that there is a greater rejection when two legs are stimulated by unacceptable compounds than when a single leg is stimulated. This apparent summation is attributable, at least in part, to the difference with respect to sucrose stimulation. Note that in one member of the pair there is sucrose on both sides of the partition while in the other it is on one side only. This result agrees with II above.

IV ($H_2O/P+s$ vs. P/s). Greater rejection is obtained when opposing compounds stimulate the same leg than when they act independently on opposite legs. This effect may be due either to some inhibiting action of the repellent compound on the sugar receptors over and above its independent effect (see I) or to some "loss" of repellent effectiveness as a result of looser central integration when opposing stimuli act on different sides.

V (P/s vs. $P/s+P$). There is greater rejection when the unacceptable compound acts on both legs instead of on one. Part of the observed difference may result from the fact that in one dish P and s are acting independently as well as contralaterally and that ipsilateral repellency is greater than contralateral repellency as shown in IV above.

VI ($P+s/P$ vs. $P+s/H_2O$). When the sugar is identical in both tests (here sucrose is on one side only), the repellency is slightly but consistently higher with the repellent on both sides of the partition. This experiment shows that for unacceptable compounds the bilateral threshold is lower than the unilateral threshold.

Unilateral versus bilateral stimulation by unacceptable compounds

As has already been pointed out, a critical comparison of unilateral versus bilateral thresholds must be based upon complete threshold frequency distribution curves and not merely upon responses to a single selected concentration. Consequently, as a check on the foregoing results, especially comparison VI, and with propanol and HCl as models, tests were run over complete concentration ranges. With propanol, tests were run with one-legged flies, two-legged flies, and with partitioned dishes. Propanol was tested with and without the addition of sucrose. With HCl, tests were run with one-legged and two-legged flies. In each case 0.1 *M* sucrose was mixed with the HCl. Data are summarized in Table I. Three facts emerge clearly from an examination of these data: (1) there is no statistically significant difference between unilateral and bilateral thresholds for either propanol or HCl; (2) nevertheless, unilateral thresholds have a slight but consistent tendency to be lower than bilateral thresholds; (3) there is no significant difference of threshold for propanol in water and in sucrose.

Discussion

As early as 1938 the experiments (Table 16) of Imamura in which tarsal receptors of the fly *Sturmia sericariae* Cornalia were stimulated by sugar had given an intimation of an increased sensitivity to bilateral stimulation over unilateral. Studies of olfactory thresholds of *Phormia regina* (Dethier, 1952a) had yielded data which suggested the possibility of a precise quantitative relationship between bilateral and unilateral thresholds. It is now clear that such quantitative relationships exist. But although it can be proven that for any individual the bilateral thresholds for certain compounds are significantly lower than the unilateral thresholds, it is by no means certain that the increased sensitivity is attributable to contralateral summation in the usually accepted sense.

In the analogous case, where comparisons have been made between monocular and binocular vision, Pirenne (1943) and Bárány (1946a, 1946b) have pointed out that the experimental procedure by its very nature assures that the two eyes will see more clearly than one. "Let us assume that the visual acuities (or other thresholds) of both eyes fluctuate independently of one another . . . and that the instantaneous thresholds for monocular vision have the same distribution in both eyes, . . . then if the one eye alone has the chance a of seeing the symbol, both eyes together have the chance $2a - a^2$. As a is smaller than 1, this expression will always be greater than a—that is to say, two eyes will be able to see better than one solely as a result of random combination" (Bárány, 1946b, p. 127). And, as Smith and Licklider (1949) go on to state, the same source of bias is inherent in the procedure as applied to the determination of thresholds in other sense modalities. The idea can be clarified still further by quoting from the study of hearing by these authors (p. 279). "In order to estimate the magnitude of the bias, it is necessary to define the null condition under which we should say that there is no binaural summation. We can imagine, for this purpose, two monaural listeners, one with only a right ear, the other with only a left ear. The two listeners have no means whatsoever of communicating with each other, but both report to the same experimenter. To obtain measures of monaural and 'binaural' sensitivity, the experimenter tests the two listeners separately (successively), then together, in the latter instance recording a positive response whenever *either* listener reports hearing the stimulus tone. It need hardly be said that we do not propose this schema as a psychophysiological hypothesis. There is of course no doubt that the two ears of a single listener do send their messages to a common center in which true summation may occur. Evidence of interaural interaction has been found in psychophysical experiments on sound localization and on masking and in electrophysiological observations at the levels of the cochlear nucleus and the temporal lobe. The schema is therefore a 'null hypothesis' [of supplementation] which a given set of data should lead us to reject before we say that the data constitute evidence of true binaural summation."

The applicability of this hypothesis of supplementation to the present case is easily tested. Let us assume that x number of sucrose receptors on the tarsi must be activated in order to insure a response, that is, that there is intra-leg summation. Then at the median acceptance threshold value 50% of the flies have x or more receptors acting. The probability at this concentration that a fly (x or more active receptors) will be in the half of the population which is responding is 50%. This probability can be raised either by increasing the concentration or by increasing the number of available receptors (n). If n is doubled, the probability of a response occurring increases. The increase can be calculated from the expression $1 - q^2$ where q is equal to the fraction not acting at the median concentration. Accordingly, the concentration which elicits a response from 50% of the one-legged flies should elicit a response from 75% of the two-legged flies if there is no interaction between the two legs. In the present experiments this expectation is realized. As a visual examination of Figure 1 shows, the response of the two-legged flies is not greater, except at the highest concentrations, than that which would be predicted on a probability basis from the behavior of one-legged individuals. The difference between the expected line (calculated from the line for one-legged thresholds from the expression $1 - q^2$ where $q =$ the fraction not responding) and the line describing actual two-legged thresholds is not significant. Thus the data do not constitute evidence of true contralateral summation. Nevertheless, as was pointed out in the case of olfactory responses (Dethier, 1952a), the results represent from the point of view of the integrated organism a behavioral summation of no little importance.

The action of unacceptable compounds (propanol, HCl, NaCl) on one-legged and two-legged flies appears at first to follow an entirely different pattern from that observed with sucrose. Bilateral thresholds are not lower than unilateral thresholds as might have been expected and as the spot tests (comparison VI) show. Actually, data from the spot tests and from complete concentration tests are in agreement, and supplementation occurs in much the same manner as it does with sucrose. The following explanation of the one-legged and two-legged fly experiments should clarify this point: (1) less sucrose is required to elicit a response from two-legged than from one-legged flies; (2) in most of the experiments with propanol and HCl, the test solutions contained 0.1 M sucrose regardless

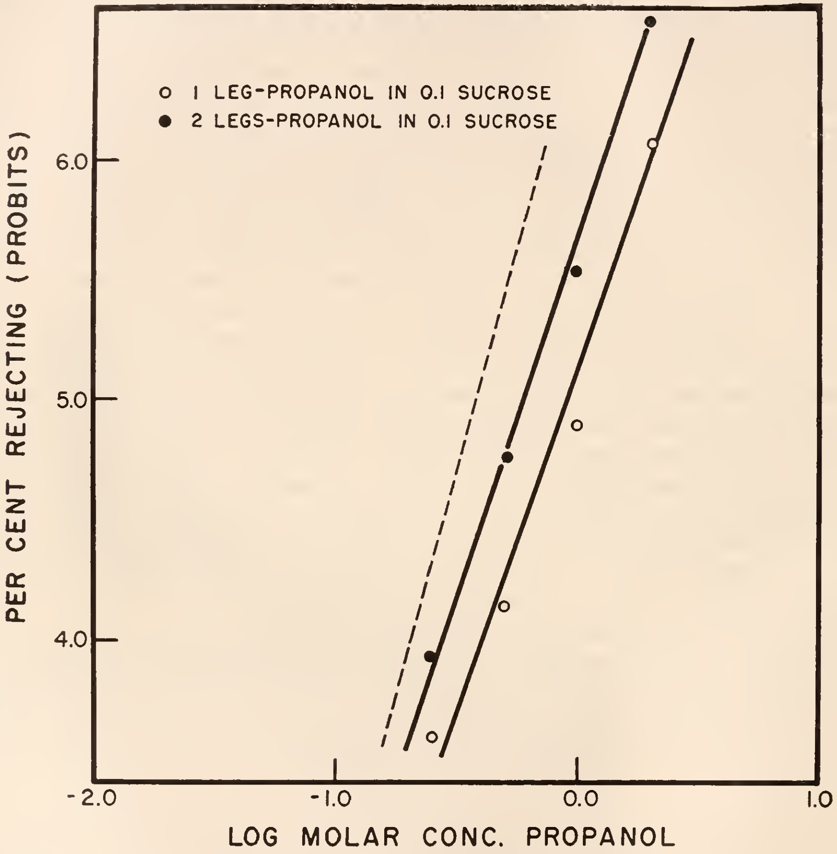

FIGURE 2. Comparison of the distribution of rejection thresholds for propanol, as a function of concentration, for flies stimulated unilaterally or bilaterally. The broken line represents the theoretical distribution of bilateral thresholds (two-legged flies) calculated from the expression $1\text{-}q^2$ where q equals the fraction of the population of one-legged flies not responding.

of the number of legs to be stimulated; therefore, since the sucrose "sums" contralaterally, there was actually a greater effect for the propanol and HCl to overcome in two-legged flies than in one-legged flies; (3) if propanol and HCl "sum" as sucrose does, the greater sucrose effect would be overcome with the same concentrations of propanol or HCl in the one-legged and the two-legged flies; in other words, the rejection thresholds should be the same regardless of the number of legs stimulated; (4) if neither propanol nor HCl "sums," then a greater concentration of these compounds would be required on a two-legged fly to overcome the increased sucrose effect, and, therefore, propanol and HCl thresholds should be higher with two-legged than with one-legged flies.

A comparison of thresholds of one-legged and two-legged flies for unacceptable compounds reveals no statistically significant difference between the two although a slight but consistent tendency for the bilateral threshold to be higher has been commented upon. It would be expected, however, that if this tendency were truly an expression of a failure to sum or supplement, as outlined under (4) in the preceding paragraph, the difference would be of the same order of magnitude as that found with unilateral and bilateral sucrose thresholds but of the opposite sign. Obviously the observed difference is too small. The alternative suggestion, that unacceptable compounds do indeed "sum" in a manner similar to sucrose, though possibly not to the same extent, fits the data with fewer contradictions. This interpretation is strongly supported by the spot tests in comparison VI ($P + s/P$ vs. $P + s/\mathrm{H}^2\mathrm{O}$) where the sugar concentrations are controlled in such a way that the difference in unilateral and bilateral thresholds shows up directly.

Furthermore, this interpretation agrees with the results obtained in olfactory tests where the unacceptable compound pentanol was found to stimulate a fly with two antennae more effectively than it did a fly with one antenna (Dethier, 1952a). The reason that a threshold difference could be demonstrated directly in the olfactory tests is explained by the fact that the pentanol vapor was not counteracting the effect of an acceptable compound. The pentanol rejection threshold represented the concentration necessary to drive the flies away from light. When one antenna was amputated in these experiments, the number of receptors being stimulated by light was in no way affected. On the other hand, immobilization of one leg in the tarsal experiments not only reduced the number of receptors being stimulated by the unacceptable compound but also the number affected by the acceptable compound.

The foregoing considerations involving tarsi have been based principally upon those cases in which the unacceptable compounds were mixed with sucrose. An examination of Table I will show that comparable results were obtained with propanol when sucrose was absent from the test solutions. Determinations of the rejection thresholds of thirsty flies for propanol in water gave unilateral and bilateral threshold values which were not significantly different from each other. By applying the same reasoning to data obtained from water mixtures as was applied above to data relating to sucrose mixtures, one arrives at the conclusion that just as there is supplementation of response to sucrose, so also is there supplementation with water. Since the concentration of water cannot be altered, direct demonstration of the phenomenon of supplementation is impossible in this case.

In the sense that propanol, HCl, and NaCl alter the normal pattern of activity from tarsal receptors, they may be said to be stimulating. While a conclusion of this sort is hardly unexpected insofar as the electrolytes are concerned, some find it difficult to envision alcohol as a stimulus to sensory structures. Yet observations of the reactions of individual flies show that in solutions containing alcohol there is agitated movement of the feet, even attempts at withdrawal, and that all gradations exist between absolute failure to extend the proboscis, complete extension followed immediately by exceptionally rapid withdrawal, and partial rather hesitant extension. In other studies, many species of insects have been shown to be stimulated by alcohols. The ovipositors (which are insensitive to water) of certain hymenopterous parasites and of the cricket (*Gryllus*) are excited by inorganic electrolytes and aliphatic alcohols. Application of threshold concentrations results in characteristic movements of the organ (Dethier, 1947, 1951). The beetle *Laccophilus* is stimulated to greater swimming activity by alcohols (Hodgson, 1951). *Phormia* is stimulated via the antennae to greater flying activity by alcohol vapors (Dethier and Yost, 1952), and *Drosophila* (Reed, 1938) is attracted by low concentrations of short-chain aliphatic alcohols. On the other hand, it is also known that short exposure to high concentrations or prolonged exposure to lower concentrations of alcohols will result in narcosis. What may be narcosis of tarsal sugar receptors has been observed in *Phormia* (cf. Dethier, 1951).

From the neurophysiological point of view the action of a compound like alcohol could be either excitatory or depressant, or excitatory at one concentration and depressant at another. Peripheral inhibition of activity in sense organs by a stimulus as a means of "stimulating" an animal has been recorded sporadically. Granit (1947) and others have described fibers of the retina in which there is commonly spontaneous activity which may be depressed by illumination. Löwenstein and Sand (1940) have reported that spontaneous discharges from single fibers from the horizontal ampulla of the isolated labyrinth of *Raja* are increased by ipsilateral stimulation and inhibited by contralateral stimulation.

Ultimately the revelation of the nature of those events which are occurring peripherally in *Phormia* at the time of stimulation can be realized probably only through the agency of electrophysiological techniques. However, the experiments reported here foreshadow to some extent the nature of these events. Data from experiments with divided dishes do not in themselves prove that unacceptable compounds and sucrose act upon different receptors, but taken in conjunction with other experiments they lend strong support to the idea that there are at least two types of tarsal chemoreceptors. Admittedly, it could be argued that only a single type of receptors need be postulated to explain the facts. An argument along these lines, which could apply equally well to ipsilateral and contralateral stimulation by opposing stimuli, would require that sucrose stimulate the receptors and that alcohol depress them. It would require further that there be spontaneous activity from the receptors and that any decrease in the basal level of discharge from one leg could offset in the central nervous system the increased activity from the opposite leg which was undergoing stimulation by sucrose. According to this scheme, however, any factor which could depress spontaneous activity in one leg should be able to prevent proboscis response when the opposite leg is stimulated by sucrose. Amputating a leg would be a most effective means of obliterating spontaneous activity. Yet, when this is done, stimulation of the remaining prothoracic leg by a threshold amount (for unilateral stimulation) of sucrose elicits the usual proboscis response.

Another experimental result which is not in accord with the one-receptor-type hypothesis is the quantitative difference between supplementation with sucrose and supplementation with unacceptable compounds. With sucrose the difference between unilateral and bilateral thresholds is considerable; with unacceptable compounds it is difficult to demonstrate, even in the absence of such disturbing influences as water and sucrose (*cf.* spot test comparison VI). It would be expected that supplementation would be of equal value with acceptable and unacceptable compounds if these were acting on the same population of receptors.

In view of the objections just stated, it is difficult to reconcile the available data with a one-type-receptor hypothesis. All of the results reported are more consistent with the idea that sucrose and unacceptable compounds act on different populations of receptors. It is to be hoped that the tarsal receptors may eventually yield to attack by electrophysiological methods.

SUMMARY

Acceptance thresholds of the tarsal chemoreceptors of the blowfly, *Phormia regina,* for sucrose and rejection thresholds for HCl, NaCl, and propanol have been determined. Comparisons were made of the thresholds of one-legged and two-legged flies. Comparisons were also made of rejection thresholds determined (a) on one leg exposed to a mixture of sucrose and unacceptable compound and (b) on two contralateral legs, one of which was exposed to sucrose alone and the other to an unacceptable compound alone, the exposure being simultaneous. The following results were obtained:

(1) The bilateral threshold for sucrose is lower than the unilateral threshold.
(2) For sucrose the decrease of bilateral over unilateral threshold is never greater than can be satisfactorily accounted for on a simple probability basis.
(3) Unacceptable compounds terminate the response to sucrose even when applied to the leg not receiving sucrose, *i.e.,* they can act contralaterally as well as ipsilaterally.
(4) There is a greater rejection when two opposing stimuli act on one leg than when they stimulate two contralateral legs.
(5) Unacceptable compounds also "sum" contralaterally.
(6) Supplementation also occurs in the response of thirsty flies to water.
(7) The prevention of proboscis extension to water and sucrose by unacceptable compounds is predominantly a central phenomenon.
(8) Available evidence favors the interpretation that there are at least two distinct sets of receptors on the tarsi, sugar receptors and non-sugar receptors.

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MATING TYPES IN TETRAHYMENA[1]

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The potentialities of *Tetrahymena* as a genetic tool have long been considered by those working with this ciliated protozoan. Its value stems from the fact that it is one of the few animal cells that can be grown in a defined medium. The failure of laboratory strains to reproduce sexually has been the chief obstacle to its use in genetic studies. The recent discovery of conjugation (selfing) in several strains (AA) taken from the Ann Arbor area (Elliott and Nanney, 1952) stimulated a further search for wild strains in which mating types might be found. These have been found and the purpose of this paper is to describe certain aspects of their physiology and cytology, and to discuss how they may be handled in the laboratory with a view to their use in genetic studies.

MATERIALS AND METHODS

One hundred twenty-seven clones of *Tetrahymena* sp. (probably *T. pyriformis*) were established from 15 different fresh water habitats in the Woods Hole, Massachusetts area (Elliott and Gruchy, 1952). Three of these turned out to be selfers whereas all others did not conjugate within the clone. Cross-matching in all combinations finally yielded a pair of mating clones which came from one pond. Sixteen of the 17 other clones from this source mated readily with either one or the other of these two. Clone 52 from another pond conjugated with both of the first two. These three have been designated WH (Woods Hole) strains 6, 14, and 52, mating types I, II, and III, respectively. Throughout this paper strains 6, 14, and 52 will be referred to only as mating types I, II, and III. Two strains which will mate with types I, II, and III have also been isolated from a lake in northern Minnesota (Park Rapids area). One of these is another mating type I which conjugates with types II and III; the other, a type II, mates with types I and III.

All clones were established in axenic cultures by placing several cells from a bacterized culture (0.1% Cerophyll seeded with *Aerobacter aerogenes*) in a depression slide containing a mixture of penicillin G and streptomycin (250 γ/ml. of each) in the stock medium. The stock medium contains Bacto-tryptone 5 gm., Bacto-proteose-peptone 5 gm., sodium acetate 1 gm., thiamine HCl 0.002 gm., yeast extract 0.1 gm., KH_2PO_4 1 gm., and 1000 ml. of glass-distilled water. Its pH is adjusted to 7.2 with NaOH. After approximately 12 hours, single-cell isolations were made into several depressions containing the antibiotic medium. Twenty-

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four hours later, during which time growth of the protozoan had occurred (about 10 divisions), the entire drop was transferred to a test tube containing the stock medium without the antibiotics. Only rarely did this procedure fail to eliminate contaminating organisms as verified by the customary sterility tests.

Stock lines have been maintained continuously in this medium and, except where otherwise stated, all experiments are conducted with organisms grown in this medium. Loop inoculations reach peak growth in about 7 days and the cells remain viable for two or more months, thus permitting the handling of large numbers of clones without frequent sub-culturing.

Experimental

I. Nutrition

In determining the nutritional requirements of the mating types, the eleven amino acids and seven B-vitamins essential for the long-established strain E (Elliott, 1949, 1950) were tested by single omissions from the complete defined medium that supports normal growth of strain E (Table I). The results of this

Table I

Defined medium

	Micrograms per milliliter
L-Arginine·HCl	150
L-Histidine·HCl·H_2O	110
DL-Isoleucine	100
DL-Leucine	140
L-Lysine.·HCl·H_2O	70
DL-Methionine	70
DL-Phenylalanine	100
DL-Serine	180
DL-Threonine	180
L-Tryptophan	120
DL-Valine	60
Asparagine	85
Dextrose	1000
Sodium Acetate	1000
$MgSO_4 \cdot 7H_2O$	10.0
K_2HPO_4	100.0
$Zn(NO_3)_2 \cdot 6H_2O$	5.0
$FeSO_4 \cdot 7H_2O$	0.5
$CuCl_2 \cdot 2H_2O$	0.5
Uracil	25.0
Cytidylic acid	25.0
Guanylic acid	25.0
Adenylic acid	25.0
Ca pantothenate	0.10
Nicotinamide	0.10
Pyridoxine HCl	2.00
Riboflavin	0.10
Folic acid	0.01
Thiamine HCl	1.00
Protogen or Thioctic acid	1 unit

series of experiments indicate no nutritional differences between the mating types (I, II, III) and strain E insofar as these nutrilites are concerned. In the light of experience with other strains it is unlikely that differences in purine, pyrimidine, and inorganic requirements would be found if sought for.

A comparative growth study of the mating types and strain E in defined medium showed remarkable similarity among the three mating types (Fig. 1). Strain E, perhaps as a result of its long maintenance in test tubes (20 years), grew faster after the third day and reached a higher maximum.

II. General morphology

Mating types I, II, and III have been identified as *Tetrahymena pyriformis* by Corliss (personal communication). There are no apparent morphological differences among the three types. All possess a single macronucleus and at least one micronucleus. The number of micronuclei varies within a single clone; for example, in one series of counts of the Type I clone the number varied as follows: 1 micronucleus, 78%; 2 micronuclei, 16%; 3 micronuclei, 3%; and 4 micronuclei, 2%. A similar range was found in Type II clones. An occasional individual without a micronucleus (about 1%) can be found in laboratory cultures but no amicronucleate clones have been established from nature.

The presence of more than one micronucleus complicates the cytological picture when the steps in conjugation are traced. Since unimicronuclear clones were desirable for cytogenetic studies of conjugation an effort was made to control the number of micronuclei within a clone.

Seventeen clones of mating types I and II were established and after 2–5 days of incubation were stained and the micronuclei counted. One hundred individuals from each clone were examined for their micronuclear number. Of the 17 clones, 7 were found to be as variable as the original stocks; 6 proved to be consistent for bimicronucleate individuals; and 4 contained only unimicronucleate organisms. These 4 clones with single micronuclei were carried through three serial transfers and, after two weeks, stained preparations were made from the last transfers None of these clones had remained constant for single micronuclei as indicated by average counts. However, no exconjugants with more than one micronucleus have been observed and, since multimicronucleate conjugants occur regularly, one of the results of the sexual process is to establish uniformity in the micronuclear number for the species.

The maintenance of a constant number of micronuclei in clone cultures seems to depend on chance alone. The presence of multi-micronucleate cells may be correlated with the formation of doublets (two-mouthed individuals) as suggested by Corliss (personal communication).

The possibility that the number of micronuclei might be influenced in some way by age of the culture was tested by staining 2-, 8-, and 40-day old cultures for examination. Data derived from such preparations gave no indication that the number of micronuclei was correlated with age.

During the first prezygotic division of conjugation, chromosomes (or chromosome aggregates) become clearly visible. There are four (or possibly five) relatively large, thick rods which presumably represent the haploid number. Because these "chromosomes" are comparatively large and so few in number when compared to most other ciliates they provide excellent material for detailed cytological studies.

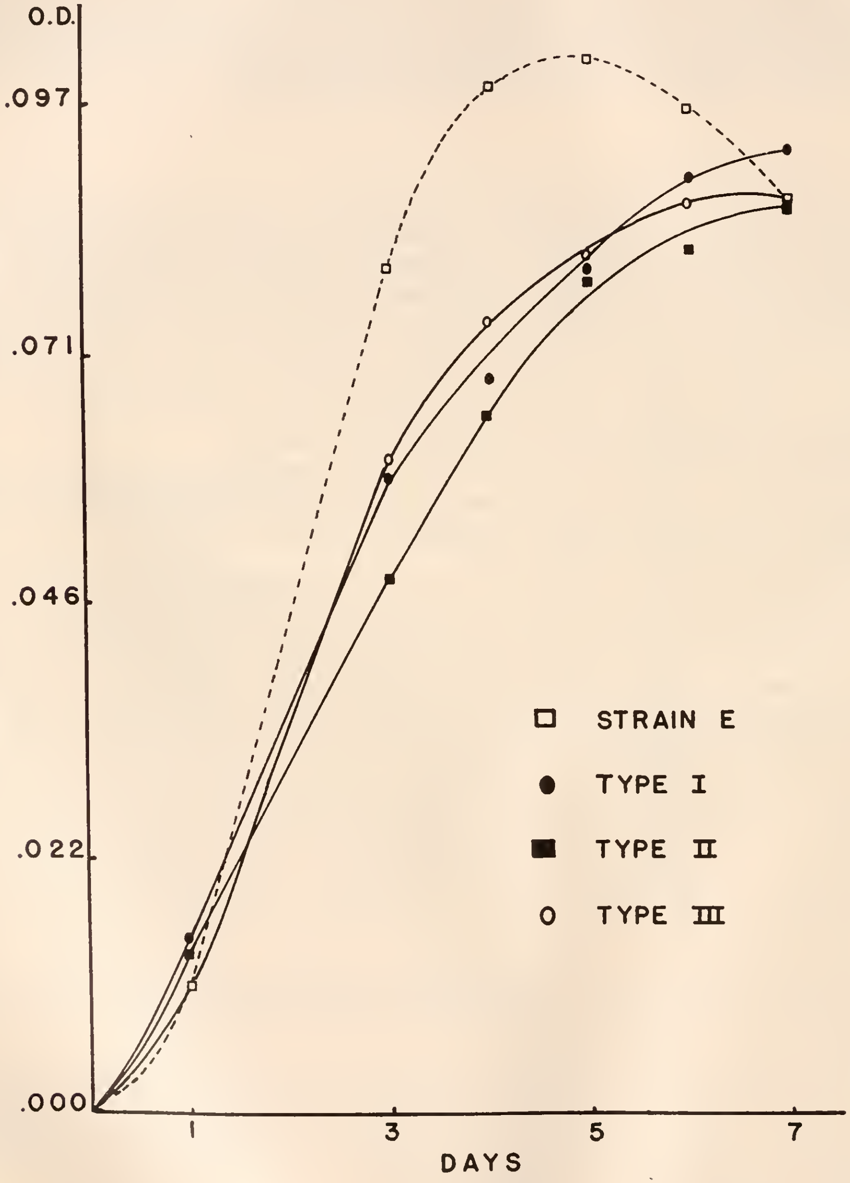


Figure 1. Comparative growth of strain E and the three mating types in defined medium. Growth as measured by optical density (O.D.) is plotted against time in days.

III. Conjugation

When washed cells of opposite mating types are mixed under appropriate conditions conjugation takes place. In order to rule out the possibility of selfing, several checks were made. By mixing a small number of cells (15–20) of one mating type with a great many (500 or more) of the opposite mating type and counting the number of pairs that appear as compared to the control in which equal numbers of cells were mixed, it was possible to prove conclusively that selfing did not occur. Furthermore, selfing has never been seen when the mating types are subjected to the same conditions that initiate the process in our selfing (AA) strains.

The preliminary mating reactions reported for some other ciliates, *i.e.*, agglutination and the formation of large clumps of animals, are absent in *Tetrahymena*. Pairs of cells are formed directly. Two animals become attached at their preoral surfaces near their anterior ends with bodies flaring at a wide angle. They are loosely attached at first and often twist and break apart. The attachment soon becomes quite firm. A conjugating pair remains active and swims as a unit with a characteristic spiraling motion.

The following description is intended to cover the normal process of conjugation in *Tetrahymena pyriformis* as seen in mating types I and II. Observations on matings between I and III, and II and III show no differences greater than the normal variation found when mating I and II. The general features of conjugation resemble those reported by Maupas (1889) for *Tetrahymena* [*Leucophrys*] *patula* (Corliss, 1953), and recently by Nanney (1953) for the selfing AA (Ann Arbor) strains of *Tetrahymena pyriformis*. In certain details the phenomenon strikingly resembles the description of autogamy in *Tetrahymena rostrata* as given by Corliss (1952a). Abnormalities, such as the occurrence of multimicronucleate conjugants and triples in conjugation, are ignored in the present paper. Also, no attempt has yet been made to work out the exact time relationships of the sequence of stages in conjugation.

A. Cytology. Cytological studies were made from material fixed in hot Schaudinn's solution and stained according to Dippell and Chao's modification of the DeLameter stain (Sonneborn, 1950). A fast green counterstain was applied in most cases. Fixed material was processed in bulk in centrifuge tubes.

The following stages are indicated diagrammatically in Figure 2. Photomicrographs of some of the stages appear in Plate I. During conjugation two cells of opposite mating types are attached only at their oral surfaces near their anterior ends.

In preconjugants and in recently attached conjugants the single micronucleus is located near the macronucleus (Stage 1). Soon after the conjugants come together the micronucleus elongates into a curved, threadlike "crescent" stage of the first prezygotic division (Stage 2). This crescent later shortens, chromosomes become visible, and the division is completed, resulting in two daughter nuclei in each cell (Stage 3). Each daughter nucleus divides again in the second prezygotic division, producing four nuclei (Stage 4). It is assumed that one of the first two divisions is reductional, as is the case in other ciliates, and that the resulting four nuclei are haploid.

Of the four nuclear products in each conjugant, only one functions in the remainder of the process. The other three eventually degenerate and disappear. The functional haploid nucleus comes in contact with the cell membrane between the conjugants where it undergoes a third prezygotic division to yield two gametic nuclei

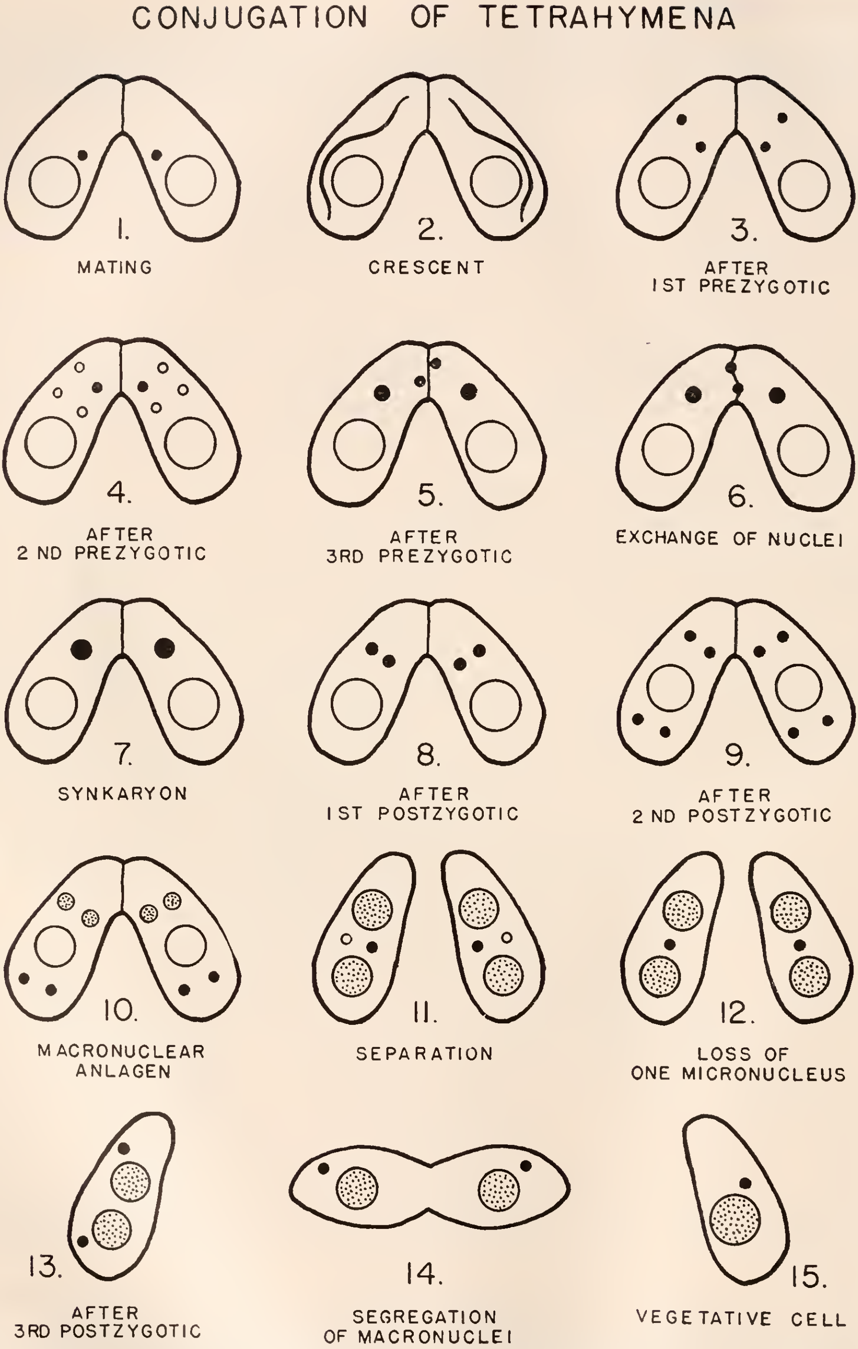

CONJUGATION OF TETRAHYMENA
1.
MATING
2.
CRESCENT
3.
AFTER 1ST PREZYGOTIC
4.
AFTER 2 ND PREZYGOTIC
5.
AFTER 3RD PREZYGOTIC
6.
EXCHANGE OF NUCLEI
7.
SYNKARYON
8.
AFTER 1ST POSTZYGOTIC
9.
AFTER 2 ND POSTZYGOTIC
10.
MACRONUCLEAR ANLAGEN
11.
SEPARATION
12.
LOSS OF ONE MICRONUCLEUS
13.
AFTER 3RD POSTZYGOTIC
14.
SEGREGATION OF MACRONUCLEI
15.
VEGETATIVE CELL

FIGURE 2. Schematic figures showing representative stages in normal conjugation of the WH strains.

(Stage 5). One of these (the stationary nucleus) takes a position anterior to the macronucleus. The other (the migratory nucleus) remains on the membrane.

In the fertilization process the migratory nucleus from each conjugant passes through the cell membrane and fuses with the stationary nucleus of the opposite cell to form the synkaryon (Stages 6 and 7).

The synkaryon then undergoes two postzygotic divisions to produce four daughter nuclei in each conjugant (Stages 8 and 9). Two of these new nuclei are located in the anterior region of the cell, and two in the extreme posterior with the macronucleus lying between. The two anterior nuclei gradually enlarge to become macronuclear anlagen which later develop into two new functional macronuclei (Stage 10). The two posterior nuclei remain small and become new micronuclei.

At about this time the conjugants separate and the old macronucleus in each exconjugant becomes smaller, more intensely stained, and spherical, eventually disappearing without fragmentation. The two anlagen become large new macronuclei and the two new micronuclei usually come to lie in the center of the cell between the macronuclei (Stage 11). One of these micronuclei degenerates, the other undergoes a third postzygotic division (Stages 12 and 13).

Each exconjugant then goes through a single fission, segregating one new micronucleus and one new macronucleus to each daughter cell (Stage 14). This fission completes the sexual process. From the original pair of conjugants four daughter cells have been produced, each of which has been restored to the normal vegetative condition with one macronucleus and one micronucleus (Stage 15).

B. Establishment of exconjugant clones. The usefulness of *Tetrahymena* as a genetic tool requires that the investigator be able to perform easily and dependably the many cross-matings involved in any genetic study with sexually reproducing laboratory animals. He must be able to mate parental types, isolate and culture exconjugant clones (F_1 generations), and then interbreed these to obtain F_2 and succeeding generations. Part of this has already been accomplished and preliminary investigations indicate that other required matings can also be made successfully.

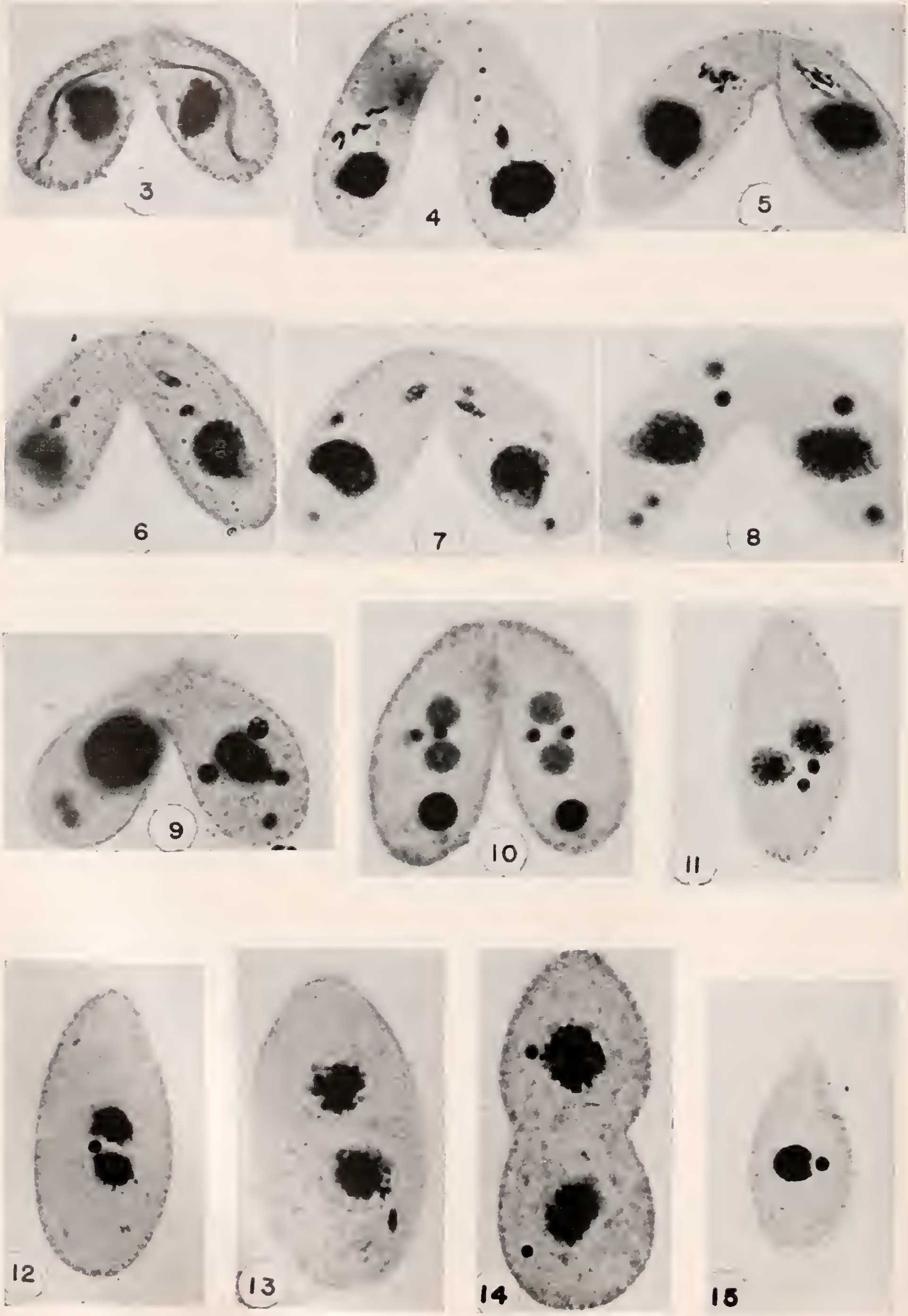
A routine procedure for obtaining exconjugant clones has been established. Since only axenic cultures of *Tetrahymena* are employed, sterile glassware and aseptic techniques are used throughout. Actively growing, young (2–7 day) stock cultures of known mating types are centrifuged and washed four times in sterile distilled water. Washed cells of two opposite mating types are mixed in shallow depression slides and allowed to stand undisturbed in distilled water for 12–24 hours. Examination under a low power microscope at intervals during this period shows the progress of pairing. It has also been found desirable to check periodically on the nuclear status of the conjugating animals. This is done quickly by staining a small sample of the cell mixture with acetocarmine and examining the wet mount under a compound microscope.

When these examinations show that many or most of the cells are paired and that conjugation is well under way, as revealed by the presence of macronuclear anlagen in many conjugants, individual pairs are isolated from the mixture with a micropipette and transferred to drops of distilled water in depression slides. These isolated pairs are then allowed to stand until the conjugants complete the sexual process and separate.

The resulting exconjugants are then picked up singly and transferred to drops of culture medium in depression slides. Within 48 hours each isolate will usually have

PLATE I

Photomicrographs of stages in conjugation of *Tetrahymena pyriformis,* mating types I and II.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divided 4–8 times and the daughter cells are then transferred *en masse* to a tube of medium to establish an exconjugant clone. This clone is maintained thereafter by routine serial transfers at weekly (or longer) intervals.

Mention was made earlier of the desirability of waiting until conjugation was nearly completed before isolating conjugating pairs. It has been found that in most cell mixtures there is a certain amount of abortive conjugation, that is, of individuals coming together but separating after a time without actually conjugating. In some cases this premature separation can probably be blamed on mechanical agitation. Conjugants are only loosely attached at first and if picked up too soon are broken apart in the pipetting process. However, there seem to be other, as yet unknown, factors involved. For no apparent reason some mixtures yield a higher percentage of exconjugants than others. For example, from 154 isolated pairs 26 exconjugant cultures (17%) were obtained. From the separate groups of 20–30 isolates making up the larger series, yields of successful exconjugant cultures varied from 0% to 38%. Some of the failures can definitely be attributed to improper handling such as pipetting too early before conjugants are firmly attached and to a failure to recover isolates. Other failures, however, seem to be due to spontaneous separation of paired individuals.

Because of this high percentage of false conjugation it has been found necessary to verify each "exconjugant" by other tests. One method used is to demonstrate new macronuclei and new micronuclei in one separated individual from each isolated pair. Acetocarmine staining clearly brings out the nuclear picture. However, it is laborious and time-consuming to isolate, stain, and locate on the slide a single organism. An easier test, and one which has proved to be just as valid, is based on the fact that exconjugant clones go through a period of sexual immaturity, that is, a period during which they show no mating reaction (see below). In practice, then,

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FIGURE 3. First prezygotic division showing micronuclei elongated into characteristic crescents (Stage 2).

FIGURE 4. First prezygotic division in which the crescents have condensed revealing three distinct "chromosomes." At least one other "chromosome" does not show in this photograph. These are presumably tetrads.

FIGURE 5. A stage slightly later than that shown in Figure 4, in which the "chromosomes" have separated and are moving to the poles in anaphase.

FIGURE 6. After first prezygotic division. One of the nuclei in the right conjugant is entering the second prezygotic division (Stage 3).

FIGURE 7. Four nuclear products of the second prezygotic division. Three relic nuclei are degenerating and one functional nucleus in each conjugant is undergoing the third prezygotic division (Stage 4).

FIGURE 8. Four nuclei resulting from the second postzygotic division can be seen in the left cell (Stage 9). This second division has not yet occurred in the right cell.

FIGURE 9. The two anterior nuclei in the right cell are enlarging to become macronuclear anlagen (Stage 10).

FIGURE 10. A later stage which shows the old macronuclei degenerating.

FIGURE 11. A recently separated exconjugant (Stage 11).

FIGURE 12. A later exconjugant stage in which one micronucleus has disappeared (Stage 12).

FIGURE 13. The functional micronucleus is undergoing the third postzygotic division. The exconjugant is now larger due to feeding.

FIGURE 14. Division of the micronucleus is complete and cytoplasmic cleavage is well underway, segregating a new macronucleus to each daughter cell (Stage 14).

FIGURE 15. Normal vegetative cell (Stage 15).

all newly established clones derived from "exconjugants" are mixed with mating types I, II, and III. If conjugation occurs, the clone is discarded. If conjugation does not take place the clone is considered a true exconjugant.

C. Factors influencing conjugation

The primary requirement for demonstrating conjugation in *Tetrahymena* is, of course, the mixing of organisms of two appropriate mating types. However, there are other factors involved which influence the mating process. As shown in the following experiments the conditions under which the cells are mixed can determine the rate at which conjugation takes place and whether or not it will occur at all.

1. Starvation. Conjugation has never been observed in the presence of nutrients, even in old cultures. However, mating can be regularly initiated by washing, which induces rapid starvation. Cells taken from any phase of the growth cycle, or those that have passed the peak growth (one to four weeks old), and washed in distilled water by centrifugation initiate conjugation at rates which are, within limits, proportional to the number of washings. The period between mixing and the appearance of the first conjugating pairs is here termed the *refractory period,* which is approximately 8 hours at room temperatures (20–22° C.) after washing once in distilled water. The refractory period is decreased to 6 hours when the cells are washed twice. Four washings reduce the time to approximately 4 hours and any more washings fail to decrease this time any further.

Additional evidence that conjugation occurs only between starved animals was obtained in the following way. Washed cells (4 times) were allowed to stand in distilled water for varying lengths of time. These starved cells decreased the refractory period at a rate which depended, within limits, on the length of time they remained in the water before mixing. For example, cells permitted to stand 24 hours in water mated in 1½ to 3 hours whereas those that starved 48 hours mated in 1–2 hours. Apparently, freshly washed cells retain sufficient reserve foods to delay mating.

Direct observation of the protozoa tends to verify this starvation requirement for mating. There is usually a definite and easily recognizable difference in appearance between cells freshly washed and those "ready" to mate. The former are large, vacuolated, and relatively sluggish whereas the starved cells, which are about to conjugate, are smaller, slender, devoid of vacuoles, and quite active.

2. Temperature. Temperature also plays an important part in the mating reaction. Several experiments were performed in an attempt to determine the optimum temperature for conjugation. Depression slides containing mixed washed suspensions of types I and II were incubated at different temperatures and examined at frequent intervals to detect the onset of conjugation. It was found, as shown in Table II, that conjugation began sooner at about 38° C. than at lower temperatures. This effect might be due to increased metabolism at higher temperatures leading to a more rapid depletion of food reserves.

3. Surface area-volume relationships. It has been observed that mating occurs slowly and involves only a few pairs in deep suspensions of washed cells. The same cell mixture will produce abundant conjugating pairs in a much shorter time when placed in shallow containers such as depression slides or watch glasses. The influence of fluid depth on the refractory period is illustrated by the following experiment.

Ten ml. of a distilled water suspension of thoroughly washed cells (types I and II) were placed in test tubes in which the fluid depth was 80 mm. and in small beakers in which the fluid depth was 8 mm. at 22° C. The refractory period was prolonged from 5–7 hours in the beakers to 22–28 hours in the test tubes.

Anoxia is possibly the reason for failure to obtain conjugation in deep containers. Aeration of deep suspensions by bubbling air through the fluid failed to speed up mating but this failure may have been secondarily induced by agitation alone.

4. *Agitation.* Mechanical agitation (shaking, stirring, etc.) of a mixture, which under quiet conditions mated normally, prevented conjugation completely. Disturbing the protozoa during mating by drawing them into and forcing them out of a pipette also stopped conjugation. It appears that they must remain relatively quiet if mating is to be successful.

5. *Age of culture.* There are visible differences between young (2–4 day) stock medium cultures and those a week or more old. During the first 4 or 5 days of incubation all organisms in the tube are suspended throughout the medium with heavier concentrations in the upper levels. Longer periods of incubation result in a gradual accumulation in the bottom of the tube of debris composed of cellular fragments and many swollen, distorted and sluggish organisms. After a month there are very few active "normal" individuals. The question as to whether or not there was any correlation between this change in appearance and activity induced by age, and the refractory period was answered in the following manner. A series of (type I and II) stock medium cultures of varying ages (3, 5, 7, 11, 13, 35 days) was centrifuged and washed through four changes of distilled water. The organisms were then mixed in shallow depression slides and examined at intervals to record the appearance of the first mating pairs. In all cases the activation period was 2–4 hours in length. Age of the culture, within these limits, has no influence on the length of the refractory period.

TABLE II

Effect of temperature on conjugation

Temperature °C.	
1–2°	No conjugation, cells spherical and motionless
23–25°	Conjugation in 6–8 hours
27–28°	Conjugation in 3–5 hours
31–32°	Conjugation in 3–4 hours
37–38°	Conjugation in 3 hours
40–42°	No conjugation
46–49°	No conjugation, cells dead in 2 hours

6. *Sexual immaturity.* All exconjugant clones of *Tetrahymena* established thus far show a temporary loss of mating type. For a certain length of time after isolation the young clone does not conjugate with other clones. This is believed to be the same period of sexual immaturity reported for some varieties of *Paramecium* and other conjugating ciliates (Sonneborn, 1939).

A total of 52 exconjugants from various matings has been isolated and maintained as clone cultures. All of these failed to conjugate with parental types I, II, or III, or with each other, for two to four weeks after isolation. Serial transfers had no influence on the duration of this period. In several cases, where tested, first transfer cultures regained mating ability at the same time as fourth, fifth and sixth transfers.

It is believed that length of the immaturity period is determined by fission rate, *viz.*, the exconjugant must undergo a certain number of cell divisions before the clone becomes sexually mature as observed by Maupas (1889) in *Tetrahymena patula.* If this is correct, cultural conditions which speed up the fission rate should correspondingly shorten the immaturity period.

Cell division and population growth are influenced within certain limits by temperature. Initial growth is faster at 32° C. than at 22° C. Also, flask cultures with larger surface areas develop heavier populations than tube cultures (Elliott *et al.,* 1952). To determine the effect of faster growth on the time required to reach maturity, parallel series of flasks and tubes of stock medium were inoculated from recently isolated immature exconjugant clones. Sets of cultures were incubated at 22° C., 25° C., and 32° C., and at daily intervals cells were removed, washed, and tested to determine their ability to conjugate with types I, II, and III. At 32° C. both flask and tube cultures did not conjugate until the 18th day after isolation, at 25° C. both flask and tube cultures matured on the 19th day. Those cultures incubated at 22° C. did not mature until the 26th day. Although these results are not conclusive, they indicate that the length of the immaturity period is correlated, in part, with fission rate. Further experiments are planned to demonstrate this correlation more precisely and to develop culture techniques which will shorten the maturation time as much as possible.

Discussion

At intervals during the long period that *Tetrahymena pyriformis,* strain E, has been grown in axenic cultures (since 1932), attempts were made to induce conjugation with other strains with the hope that, should this be possible, its genetic system might be studied. Unfortunately, conjugation was never observed in any of the long established strains (E, W, GL, H) when mixed in all combinations (among themselves and with mating types I, II, and III) and under conditions which normally induce conjugation in other ciliates. Failure may be attributed to the fact that none of them possesses micronuclei. However, amicronucleate ciliates have been known to conjugate. Schwartz (1939) observed mating in *Paramecium bursaria* between experimentally enucleated individuals and normal cells. Diller (1936) reported conjugation between micronucleate and amicronucleate races of *P. aurelia.* From this evidence it seems possible that conjugation can be obtained in some of the many amicronucleate strains of *T. pyriformis* by mixing them with micronucleate strains although this has never been observed in several hundred trials. However, information reported here seems to point to the fact that there exist in nature other mating types and other varieties of *T. pyriformis.* If and when these are found, some of the problems concerning genetics and the significance of amicronucleate races may be solved.

Long ago Maupas (1889) described the cytological details of conjugation in a number of ciliates, among them *"Leucophrys patula,"* a species recently transferred to the genus *Tetrahymena* (Corliss, 1952b, 1953). More recently Horn (1951) observed mating in a strain of *Tetrahymena pyriformis.* Corliss (unpublished work) has studied conjugation in strains of *Tetrahymena* [*Glaucoma*] *parasitica* (personal communication). All of these workers were using bacterized cultures and they observed only selfing in which no exconjugants survived. The strains of selfers isolated from the Ann Arbor region (AA strains) mate readily under axenic conditions but they, too, fail to survive the process. The widespread occurrence of a sexual phenomenon so deleterious to the survival of a species as selfing seems to be, at least as it occurs under laboratory conditions, stimulated a search for conjugating strains with surviving progeny. Assuming that conjugation is beneficial to a species it is reasonable to expect that somewhere in nature mating types of *Tetrahymena* exist. The success of this search was enhanced by the fact that the mating types described here do conjugate readily under axenic conditions. Had this not been the case any genetic studies involving nutrition would have been greatly handicapped if not impossible.

It is well known that nutritional conditions of the medium are important in the sexual behavior of ciliates. For example, Chatton and Chatton (1929a) found that *Glaucoma scintillans* underwent conjugation only in the presence of certain bacterial metabolic products while Seckbach (1948) found that bacteria were unnecessary when rye grain, liver, and intestinal mucosa extracts were used. In the present investigation it is possible to control the nutrition in all respects which emphasizes the usefulness of *Tetrahymena* for genetic studies.

The nutrient requirements, insofar as the amino acids and B-vitamins are concerned, are identical for the three mating types and strain E. This is not surprising in view of the remarkable consistency in the requirements of the long established strains. Kidder and Dewey (1945) were able to show some differences in carbohydrate fermentation among several strains of *Tetrahymena pyriformis* (H, E, T-P, T, W, GHH) and *T. vorax* (V_2, PP). With the exception of strains W and GHH, which grew without serine, all required the eleven amino acids that are also essential for the mating types. Likewise, the vitamin requirements of the mating types correlate closely with that of the long established strains. Until more information is available for other closely related ciliates it is impossible to conjecture how universal these requirements are.

The conjugation process in *Tetrahymena* agrees with the generalized sequence of nuclear and cytoplasmic events established for other ciliates. It involves a temporary pairing of two animals, three prezygotic divisions of the micronucleus producing haploid gametic nuclei in each conjugant, mutual exchange of gametic nuclei and their union to form a synkaryon, three postzygotic divisions of the synkaryon, and subsequent reorganization stages of the exconjugants. The most significant difference between the mating types of *Tetrahymena* and the "selfers" described by Maupas (1889), Horn (1951) and Nanney (1953) lies in the fact that viable exconjugant clones are produced. Selfing seems to be a lethal process, whereas exconjugants from the Woods Hole strain undergo a third postzygotic division of the functional micronucleus and divide to produce viable progeny.

In reviewing the literature concerning conjugation and the mating reactions of ciliates one is impressed in many cases with the extreme periodicity or irregularity of the process. Whether or not conjugation takes place when two appropriate cultures are mixed seems to depend on a number of exacting environmental and physiological conditions. The induction of conjugation in *Tetrahymena* seems remarkably easy and simple by contrast.

Wichterman (1953) reported that time of day had a pronounced influence on the mating reaction in *Paramecium bursaria.* With a few exceptions mating does not occur in the early morning hours or after 5 or 6 P.M., and the reaction is greatest at 12 o'clock noon. Similar diurnal periodicities were found by Jennings (1939) in his varieties of *P. bursaria* and by Sonneborn (1939) in some varieties of *P. aurelia.* Conjugation has been obtained in *Tetrahymena* at any time of day or night. There is no evidence of a diurnal periodicity of any sort, or of sexual inhibition by light or darkness.

The effect of temperature on conjugation has also been investigated by several workers. Sonneborn (1939) found striking varietal differences in this regard with *P. aurelia.* Although mating types of variety 1 will conjugate at any temperature within the range examined, 9° C. to 32° C., mating types of variety 2 will not react above 24° C., and types of variety 3 not above 27° C. Strong mating reactions in *P. caudatum* at temperatures ranging from 18° C. to 24° C. were reported by Gilman (1939). Giese (1939) in a study of temperature effects on conjugation in *P. multimicronucleatum* observed that animals grown at 30° C. seldom were found to mate but did so when placed at lower temperatures. *Tetrahymena* conjugates readily at all temperatures within the range 17° C. to 38° C.; temperatures below 17° C. have not been investigated thoroughly. The principal influence of temperature seems to be on the "refractory period." Mating takes place sooner at 38° C. than at lower ranges.

There are scattered reports in the literature of the influence of certain chemical factors and pH on conjugation in ciliates. For example Sonneborn (1939) reported that the mating reaction in *P. aurelia* is weak or lacking when "deleterious bacteria," or presumably, metabolic products of these bacteria, injure the paramecium. Chatton and Chatton (1929a) studied a variety of chemical compounds, including $CaCl_2$, $FeCl_3$, pyruvic acid, glucose, and bacterial metabolic products as "zygogenic agents" influential in initiating conjugation of *Glaucoma scintillans.* The zygogenic effects of these compounds were re-investigated by Seckbach (1948) who added salts of barium and magnesium to the list of factors essential or stimulatory for conjugation in this species. Wichterman (1953) found that pH within the range 6.0 to 8.0 had little influence on conjugation of *P. multimicronucleatum.* Since we have been able to obtain conjugation so readily in *Tetrahymena* simply by mixing washed mating types in distilled water a search for specific chemical factors has not been made. The pH of unbuffered water suspensions in which conjugating pairs are found varies from 6.6 to 7.7.

The nutritive state of the protozoan seems to be an important factor influencing conjugation in all ciliates. Maupas (1889) lists hunger, sexual maturity, and diverse ancestry as the three conditions necessary for mating in ciliates. Sonneborn (1939) observed that in *P. aurelia* the mating reaction does not take place in cultures that are either over-fed or completely starved. A similar situation exists in *P. caudatum* (Gilman, 1939) and *P. bursaria* and *P. calkinsi* (Wichterman, 1953). Giese (1939) found that food appeared to be the most important single factor in regard to conjugation in *P. multimicronucleatum* and that a decline in available food after a period of plenty was required. Chatton and Chatton (1929b) stated that starvation was a necessary condition for conjugation of *Glaucoma scintillans.* Evidence obtained thus far on the effect of starvation on conjugation of *Tetrahymena* agrees in general with that reported for other ciliates.

Tetrahymena must be at least partially starved before it will conjugate. Unlike those ciliates which seem to require a nutritional state intermediate between well-fed and completely starved, *Tetrahymena* has been found to conjugate normally even after starving in distilled water for 3 or 4 days.

One of the three conditions necessary for conjugation in ciliates, as cited by Maupas, is sexual maturity. Observations by many investigators indicate that in some species, at least, conjugation can be induced only when the animals are sexually mature. According to Calkins (1933) *Uroleptus mobilis* will mate only after a period of from 5 to 10 days following fertilization. Many races of *P. aurelia* (Sonneborn, 1939) do not conjugate during the first week or two; other races, however, lack this period of immaturity and are able to mate again immediately following conjugation. Jennings (1939) reported a state of sexual immaturity for *P. bursaria* ranging from a few weeks to several months. Periods of immaturity have been found in *P. caudatum* by Gilman (1939) and in *Euplotes* by Kimball (1939). *Tetrahymena* also goes through a definite period of immaturity during which exconjugants are not sexually reactive for a week or more. It may be possible to correlate this period with number of fissions.

From the observations reported here it seems quite clear that *Tetrahymena* has potentialities as a tool for further investigations in protozoan genetics and should supplement the already voluminous literature on *Paramecium* and other ciliates. Moreover, because its biochemistry and physiology are well known, it should become an important addition to the list of microorganisms that have already greatly enhanced our knowledge of biochemical genetics.

SUMMARY

1. Conjugating strains representing three different mating types of *Tetrahymena pyriformis* have been isolated from fresh water ponds in the Woods Hole, Massachusetts, area and established in axenic cultures.

2. These strains have the same amino acid and vitamin requirements as *T. pyriformis E.*

3. The cytology of conjugation is described.

4. Routine laboratory procedures for obtaining conjugation and establishing exconjugant clones (F_1 and successive generations) are presented.

5. Some of the factors influencing conjugation in *T. pyriformis* (nutritive state, temperature, oxygenation, agitation, culture age, and sexual maturity) are discussed and compared with reports in the literature dealing with conjugation in other ciliates.

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THE RELATION OF BODY WEIGHT TO BODY SURFACE AREA IN MARINE FISHES

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In the course of attempting to establish a satisfactory basis for comparison of gill areas of certain marine fishes, considerable information about the body surface area has accumulated. Much has been written on the value of knowing the area of the body surface in metabolism studies and the difficulties in obtaining it. The formula most commonly used for determining surface area when the weight is known is $S = K w^{2/3}$, where S represents the area of surface, w is the body weight, and K is a constant for the particular species. Reported values for K have varied from less than 5 to more than 18 for different species. Benedict (1938), who has carefully reviewed at length his own work and that of others as it relates to metabolism and body surface, believes that with the exception of animals that tend to become spherical or greatly elongated, the value of K is in the neighborhood of 10. He gives 10 as "the best value of K" for 21 of 30 birds and mammals, with a range 9 (mouse, guinea pig) to 11.8 (macaque). He further points out, however, that there have been wide discrepancies in the determination of body surface area, even for the same species. More recently Zeuthen (1947) and Kleiber (1947) have also reviewed the subject and have called attention to variations and discrepancies reported in the literature. Kleiber (1947) recommends the use of the ¾ power of the weight as representative of metabolic size.

It is not the purpose here to enter into a discussion of metabolic rate, but rather to make available data concerning the relation of body weight to body surface in certain fishes.

This work was carried out at the Marine Biological Laboratory, Woods Hole, Mass., and at the Duke University Marine Laboratory, Beaufort, N. C., and was supported in part by the Duke University Research Council.

Methods of Obtaining Body Surface Area

A few trials quickly demonstrated that the use of 10 as a value for K in the formula $S = K w^{2/3}$, while giving approximately accurate determinations of body surface area in some fishes, produced wide deviations from experimentally obtained surface areas in other species. Measurements of body surface area were made directly on over 300 individual fish representing 39 species and 25 families. From these observations average values of K for the different species were determined.

Most data were obtained by following the method of Riess (1881), who determined the surface area of pike by drawing around the body, allowing for depth and omitting fins, and then calculating the area of the outline drawing. This procedure proved quite satisfactory for most of the fishes studied and particularly so for the greatly depressed or compressed species, such as flounders, butterfish, and cutlass fish. That greater difficulty was encountered in employing the method on odd-shaped fishes like the puffer, sea robin, and toadfish is reflected in the wider variations of individual determinations on these species.

A few attempts were made to determine the area of the removed integument of puffers but the skin stretched so readily that it was difficult to obtain consistent results and this method was abandoned.

Until some degree of proficiency was obtained in estimating the depth to allow in making outline drawings of the body of odd shaped fishes, the area of the outlines was checked by the more tedious and laborious method of covering the body with pieces of paper and obtaining the total area of the various pieces. The area of all outlines was secured by use of a planimeter.

RESULTS AND DISCUSSION

The values of K in the formula $S = K w^{2/3}$ for 39 species of fish are shown in Table I. It can be clearly seen that if the formula is to be used for the determination of body surface area in fishes, the value of K must be established for each species, or at least for each type of body plan. The more or less rotund species have low values for K while the greatly depressed and compressed species have high values.

TABLE I

Values of the constant K for marine fishes in the formula $S = Kw^{2/3}$, where S represents the area of body surface and w is the body weight

Family	Species of fish	No. of determinations	Weight in grams			Value of K		
			Max.	Min.	Ave.	Max.	Min.	Ave.
Clupeidae	Menhaden, *Brevoortia tyrannus*, adults	36	729	382	553	8.3	7.1	7.8
Clupeidae	Menhaden, small	10			19			8.7
Clupeidae	Thread herring, *Opisthonema oglinum*, small	4	46	40	43			8.7
Anguillidae	Common eel, *Anguilla rostrata*	4	562	94	345	11.8	10.7	11.3
Leptocephalidae	Conger eel, *Leptocephalus conger*	1	3519	3519	3519	10.2	10.2	10.2
Pleuronectidae	Fluke, *Paralichthys dentatus*	7	524	86	401	12.2	11.6	11.8
Pleuronectidae	Sand flounder, *Lophopsetta maculata*	1	395	395	395	15.3	15.3	15.3
Pleuronectidae	Winter flounder, *Pseudopleuronectes americanus*	2	745	723	734	14.7	14.6	14.6
Trichiuridae	Cutlass fish, *Trichiurus lepturus*	12	131	47	74	13.0	12.2	12.7
Scombridae	Common mackerel, *Scomber scombrus*	23	226	86	155	7.6	6.7	7.2
Scombridae	Bonito, *Sarda sarda*	2	2387	1998	2192	7.0	6.4	6.7
Scombridae	False albacore, *Gymnosarda alleterata*	1	2079	2079	2079	6.9	6.9	6.9
Scombridae	Spanish mackerel, *Scomberomorus maculatus*	9	483	39	234	8.9	7.6	8.3
Mugilidae	Jumping mullet, *Mugil cephalus*	5	250	132	165	8.8	7.5	8.0
Stromateidae	Harvest fish, *Peprilus alepidatus*	11	77	14	24	9.2	8.4	9.1
Stromateidae	Butterfish, *Poronotus triacanthus*	25	261	23	112	9.2	8.1	8.5
Carangidae	Big-eyed scad, *Selar crumenophthalmus*	3	55	52	53	7.2	6.7	7.0
Carangidae	Crevalle, *Caranx hippos*	10	155	80	120	8.8	7.6	8.0
Carangidae	Hard-tail, *Caranx crysos*	3	297	271	287	7.7	7.2	7.4
Centrolophidae	Rudderfish, *Palinurichthys perciformis*	6	294	106	199	7.8	7.1	7.5
Pomatomidae	Bluefish, *Pomatomus saltatrix*	4	1035	184	417	7.8	7.8	7.8
Coryphaenidae	Dolphin, *Coryphaena hippurus*, male	3	7033	2268	4258	9.7	9.2	9.4
Coryphaenidae	Dolphin, female	2	3289	165	1727	8.9	8.7	8.8
Moronidae	Striped bass, *Roccus lineatus*	4	2482	917	1648	8.8	7.5	8.0
Serranidae	Sea bass, *Centropristis striatus*	3	287	230	257	8.3	7.7	8.0
Pomadasidae	Pig fish, *Orthopristis chrysopterus*	1	60	60	60	7.9	7.9	7.9
Sparidae	Scup, *Stenotomus chrysops*	13	581	185	361	8.2	7.0	7.7
Sparidae	Pinfish, *Lagodon rhomboides*	4	58	49	53	8.0	7.0	7.5
Sparidae	Sheepshead, *Archosargus probatocephalus*	15	5216	54	1021	10.0	6.7	8.1
Scienidae	Spot, *Leiostomus xanthurus*	2	111	53	82	8.2	7.7	8.0
Scienidae	Croaker, *Micropogon undulatus*	4	62	40	48	8.1	8.0	8.0
Scienidae	Silver perch, *Bairdiella chrysura*	2	86	40	63	7.8	7.6	7.7
Otolithidae	Sea trout, *Cynoscion regalis*	6	1055	705	807	9.5	7.8	8.6
Ephippidae	Spadefish, *Chaetodipterus faber*	3	400	125	239	8.3	8.0	8.1
Triglidae	Brown sea robin, *Prionotus carolinus*	4	337	155	221	9.3	6.8	8.3
Triglidae	Red sea robin, *Prionotus strigatus*	10	1178	146	365	11.4	6.5	8.2
Labridae	Tautog, *Tautoga onitus*	6	1025	412	580	7.9	7.0	7.6
Tetraodontidae	Puffer, *Spheroides maculatus*	17	440	132	257	9.0	6.8	7.8
Diodontidae	Bur fish, *Chilomycterus schoepfi*	1	313	313	313	6.3	6.3	6.3
Batrachoididae	Toadfish, *Opansus tau*	58	776	15	233	11.5	6.3	8.9
Lophidae	Goosefish, *Lophius piscatorius*	2	4583	518	2551	9.5	8.8	9.2

Fusiform and moderately compressed species are intermediate in position. Of the species listed in Table I it is perhaps to be expected that the bur fish would have the lowest value for *K*, for this is a semi-rotund, short-bodied species. Highest values of *K* were recorded for the greatly depressed flounders. The value of *K* bears a relationship to habitat and taxonomic position only insofar as shape and relative weight are correlated with habitat and taxonomic position. The *K* value of fast-swimming pelagic fishes would not be at either extreme, for fast swimmers carry neither excess weight nor excess surface, as is often tolerated by bottom dwelling species.

It has been suggested that a puffer, when swelled to the maximum and thus practically spherical, would have the minimum value for *K*. This might be true if the fish filled up with water, but of course would not be true if puffed with air, for in the latter case the body surface would be increased without materially increasing the body weight. As a matter of curiosity an attempt was made to determine the surface area of a puffer both before and after swelling. As nearly as could be determined the area of the puffed surface was approximately double that of the unpuffed condition.

None of the fish used was taken during the breeding season and consequently the extra weight of ripe gonads was not a factor. With the exception of the dolphin no noticeable differences were detected between males and females. Dolphins exhibit marked sexual dimorphism and in this case males showed a significantly higher value for *K*. In other species males and females were lumped together.

Typical curves showing the relation of body surface area to body weight are presented in Figures 1 and 2 for the butterfish, mackerel, menhaden and toadfish. Comparison of the observed body surface area curves with area curves calculated on the basis $K = 10$ shows graphically that it is misleading to assume that the value of *K* is the same for all species. This is especially apparent for the larger mackerel (Fig. 1) and menhaden (Fig. 2), but is much less obvious for the toadfish (Fig. 2) where the observed value of K (8.9) more nearly approaches 10. There is also the possibility that the value of *K* is not the same for a species at all ages. Ten young menhaden averaging 19.3 grams in weight had a mean *K* value of 8.7 while for adult menhaden, weighing from 450 to 650 grams, the mean value for *K* was 7.8. The young menhaden exceeded the maximum value of *K* for adults. There is the pos-

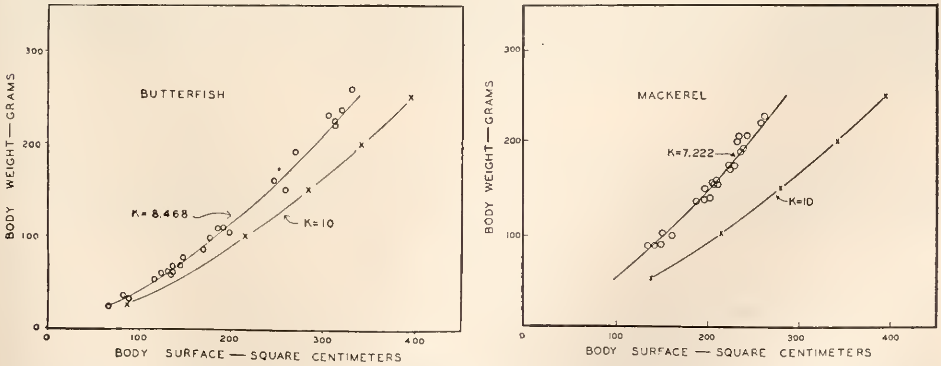

FIGURE 1. Relation of body surface area to body weight in butterfish (*Poronotus triacanthus*) and mackerel (*Scomber scombrus*). For comparison with observed values area computed on the basis $K = 10$ is also shown.

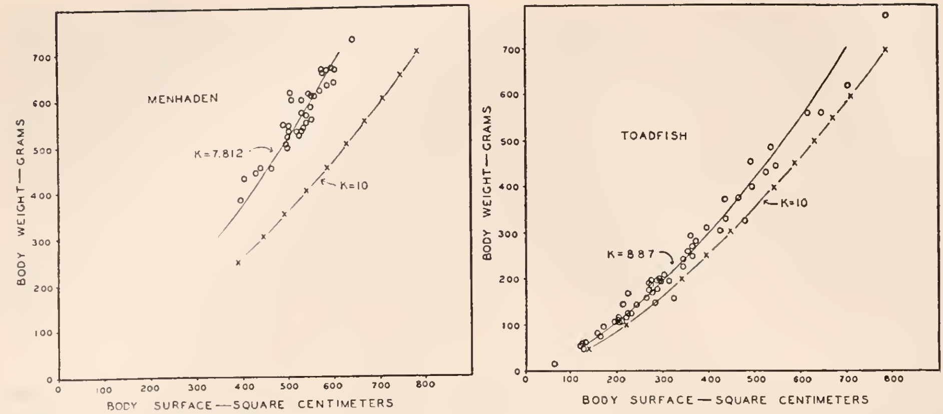

FIGURE 2. Relation of body surface area to body weight in menhaden (*Brevoortia tyrannus*) and toadfish (*Opsanus tau*). For comparison with observed values area computed on the basis $K = 10$ is also shown. The curves of Figure 2 are not on the same scale as those of Figure 1.

sibility in this case that the difference may be accounted for by error in the technique of determining the surface area of the small fish; but it seems more probable to assume that the difference in K value is due to differences in body proportions. Young menhaden are shorter bodied in relation to depth and are more compressed than adults. Four small thread herring of approximately the same shape as the small menhaden also showed higher values for K than did the adult menhaden.

In the surface area determinations on fishes the fins were excluded. Fish fins are modified in many ways and were they included as part of the body surface, the body area determinations would be misleading. For example, it was found experimentally that if the surface area of the pectoral fins of the red-winged sea robin was included as part of the body surface area, the value of K would rise to 17 instead of 8.2 when fins are omitted.

The magnitude of the deviations from the mean in the toadfish (Fig. 2) indicates the difficulty in accurately determining the surface area of some species. This raises the question of the value, in many cases of marine fishes, of expressing metabolism in terms of surface area as opposed to expressing it in terms of body weight, which can be so much more accurately and quickly determined.

SUMMARY

The value of K in the formula $S = K\,w^{2/3}$ has been determined for 39 species of marine teleosts representing 25 families. The values of K are in general higher for depressed and compressed species than for more streamlined fusiform fishes.

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A STUDY OF ISOTONIC SOLUTIONS FOR THE ERYTHROCYTES OF SOME MARINE TELEOSTS AND ELASMOBRANCHS

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The assumption is commonly encountered that in all teleosts the osmotic pressure of the blood is equivalent to about 0.25 molar (M) NaCl and in all elasmobranchs to about 0.5 M NaCl (*cf.*, Baldwin, 1949; Davson, 1951; Prosser *et al.*, 1950; Heilbrunn, 1952). The evidence for these values was obtained by the determination of the bloods' freezing point depression, from which the osmotic pressure was calculated (Botazzi, 1897; Fredericq, 1901; Garrey, 1905; Rodier, 1899; Schlieper, 1930; Smith, 1932; Krogh, 1939). The number of species examined in each class was generally small and no special significance was attributed to the observed variations in blood osmotic pressure between different species.

In addition, few attempts have been made to develop solutions for fish tissues which were balanced with respect to both ion content and concentration. Those of Baglioni (1906) and Fry (1909) were based upon the urea analyses of elasmobranch blood by Schraeder (1890); that of Lutz (1930) upon the data of Smith (1929). The Baglioni and Lutz solutions were used in studies on the elasmobranch heart, while Fry's was used in a study of blood coagulation. The "Manual of Formulae and Methods (III)" of the Marine Biological Laboratory, Woods Hole, lists a few artificial solutions supposedly "physiological" for fish tissues. Our findings reported below indicate that these solutions are not isotonic for red cells of the species studied. Apparently no systematic studies have been undertaken to determine isotonic solutions for fish erythrocytes. Solutions calculated from ion analyses of body fluids, such as those of Smith (1929) cannot, without experimental study, be taken as isotonic for red cells.

We have found that a solution of NaCl which was isosmotic with a sample of fish serum, as determined by the depression of the freezing point, was hypertonic to the red cells of that fish. This general finding led us to investigate other methods for determining solutions which were isotonic for the red cells of teleost and elasmobranch bloods.

MATERIALS AND METHODS

Blood was collected from the elasmobranchs by syringe from either the caudal vessels or the heart. In the teleosts blood was taken from the heart. In the early work, blood from mackerel and tautog was obtained from the severed branchial arteries. When a sample of blood from a single fish was less than 10 ml., blood from several fish of the same species was usually pooled for study. The blood was defibrinated by gentle stirring with a rubber-tipped glass rod. In a few cases sodium oxalate was used as an anticoagulent instead of defibrination. The blood was filtered through gauze to remove small clots and, if not immediately used, was stored at 7° C. All measurements were made at about 23° C. with blood that was less than eight hours old. Blood was collected from all of the fish immediately after their removal from sea water. In most cases the fish were caught in a fish weir.

Four separate experimental approaches were used to investigate either cell volume changes or the osmotic concentration of the serum. These four were: (1) cryoscopy, (2) hematocrit, (3) hemoglobin concentration and (4) densimetry.

Cryoscopy, the only method which does not measure cell volume, was used to determine the osmotic concentration of serum and of equivalent NaCl solutions. This method is only useful in determining isosmotic solutions, not isotonic solutions.

Measurements of cell volume by hematocrit, hemoglobin concentration and densimetric methods were based on the assumption that cells are at osmotic equilibrium with their environment, *viz.*, serum. In addition these three methods depended upon the cells behaving as osmometers (*cf.* Lucke and McCutcheon, 1932). Confirmatory evidence for this idea is found in our experiment where the relative volume of fish red cells plotted against the reciprocal of the saline concentration of the suspension medium gave a linear relation. The concentration of NaCl in which the cells maintained the same volume as in serum was taken as isotonic.

TABLE I

Freezing point depressions of NaCl solutions

Solution of NaCl in moles per liter	ΔT_f
0.05	−0.197
0.10	−0.372
0.15	−0.576
0.20	−0.771
0.25	−0.943
0.30	−1.133
0.35	−1.328
0.40	−1.449
0.50	−1.816
0.75	−2.690
1.00	−3.692

The freezing point depression (ΔT_f) of serum or whole blood was measured with the standard Beckman apparatus by the method of Findlay (1941). The ΔT_f of a series of NaCl solutions of known molarity was also determined by the same method. The NaCl used in preparing these solutions was Merck reagent grade sodium chloride "for biological work" (Williams and Jacobs, 1931). From these data a calibration curve of ΔT_f versus known *molarities* of NaCl was prepared, and isosmotic solution of NaCl interpolated from this curve from the ΔT_f measurements of fish sera. Table I presents the data from which this curve was constructed. Our NaCl solutions were unbuffered and well-aerated.

The hematocrit method of Parpart and Ballentine (1943) was used to compare the volume occupied by a known quantity of red cells in a NaCl solution of known concentration. By the use of this method one was able to determine the particular NaCl solution in which the cells occupied the same volume as in serum.

Since the loss of hemoglobin from red cells is considered an all-or-none phenomenon (Parpart, 1931), the amount of hemoglobin in a given volume of cells can be used as a measure of the quantity, *i.e.,* volume, of red cells present in a suspension (Parpart and Green, 1951). In practice, red cells in either serum or NaCl solutions were packed at 15,000 G for 10 minutes. Twenty cmm. of packed cells were hemolyzed in a given volume of 0.03 *M* NaCl containing 0.003 *M* phosphate buffer at pH 7.4. The concentration of hemoglobin was measured spectrophotometrically at 540 mμ. NaCl solutions which consistently maintained red cells at a volume, such that the hemoglobin concentration of packed samples was equal to the hemoglobin concentration of packed samples of cells in serum, were considered isotonic with serum.

The densimeter technique of Parpart (1935) was used to record rapid volume changes in red cells. This technique, which automatically records the amount of light transmitted through a red cell suspension, is capable of following volume changes of cells exposed to suspension medium from the first second onwards. By the use of the densimeter, NaCl solutions were found in which the volume of the cells was maintained constant and equal to that in serum. Since this instrument permitted the rapid determination of isotonic NaCl solutions, it was used chiefly in survey studies of a number of fish bloods.

TABLE II

Molar values of isotonic NaCl solutions
Species of fish

Method of determination	Mackerel			Tautog			Smooth dogfish					
	No. of fish	No. of dtm.	Value moles NaCl	No. of fish	No. of dtm.	Value moles NaCl	No. of fish	No. of dtm.	Value moles NaCl	No. of fish	No. of dtm.	Value moles NaCl-Urea†
Hematocrit	67	10	0.27±.007	21	7	0.21±.007	11	5	0.34±.008	14	7	0.47±.004
Hemoglobin	41	6	0.26±.005	6	3	ca. 0.23	10	4	0.35±.02	12	5	0.51±.009
Densimeter	67	10	0.26±.004	23	8	0.26±.007	13	6	ca. 0.53	14	7	0.52±.005
ΔT_f*	59	9	0.30±.004	23	8	0.27±.008	13	6	0.53±.003	13	6	0.53±.003

* Isosmotic solutions.

† Equal molecular amounts of NaCl and urea expressed as equivalent moles of NaCl.

RESULTS AND DISCUSSION

Table II presents values of isotonic solutions of NaCl for the red blood cells of mackerel, tautog and dogfish obtained by three of these methods. Also included in this table are isosmotic values for these cells as determined by ΔT_f measurements of their sera. The measure of variation is indicated by the standard error.

No significant differences in tonicity of NaCl solutions for a given species were obtained by the use of hematocrit and hemoglobin methods. Both of these methods measured the equilibrium volume attained by the cells after one hour of exposure to serum or NaCl solutions. Of the three species studied by these two methods, each would appear to require isotonic NaCl solutions of differing molarity. Statistically the differences between 0.27 *M* for mackerel, 0.21 *M* for tautog and 0.34 *M* NaCl for dogfish are real.

The densimeter measurements of isotonicity for these three species are not in complete agreement with those obtained by hematocrit and hemoglobin methods. The most unsatisfactory measurement is that for tautog blood. We do not know why this value is high or, perhaps, why the hematocrit-hemoglobin values are low. If the hematocrit and hemoglobin values are low, the cells may have decreased their internal osmotic pressure. This is possible because these methods require a longer time to determine the isotonicity (between one and two hours) than does the densimeter method, which makes such measurements within a few minutes.

When dogfish red cells are taken from serum and placed in a NaCl solution which does not contain urea, urea diffuses from the cells and within nine minutes a new equilibrium volume is reached. But when the initial volume of dogfish red cells is obtained by use of the densimeter, by using only the first 10 seconds of exposure, it is found that a NaCl solution of 0.53 *M* is "isotonic." This is because no water exchange is possible now and the only volume change which will be observed will be shrinking as urea diffuses out of the cells. This volume change due to the outward diffusion of urea was not noticeable under our conditions of measurement until about 30 seconds after the cells were exposed to the 0.53 *M* NaCl solution. Thus the apparent discrepancy in isotonic volumes for dogfish red cells between densimeter and hematocrit measurements reflects the loss of urea in the latter. The urea plays no role in determining the final equilibrium volume of these cells as was shown by Green and Hoffman (1951).

The NaCl-urea solution indicated in Table II contained both urea and NaCl in 0.35 *M* concentrations. Such a mixture is equivalent osmotically to 0.53 *M* NaCl. Aqueous dilutions of this NaCl solution expressed as the equivalent moles of NaCl appear in Table II as isotonic solutions for dogfish red cells. The isotonic value of 0.47 *M* obtained by the hematocrit method is significantly different from that arrived at by other methods. This variance is not understood. The value of using NaCl-urea solutions is to prevent urea-containing cells from undergoing any volume change. The results shown in Table II indicate that the NaCl-urea solution, containing 0.35 *M* of each of these reagents, is approximately isotonic.

The column headed ΔT_f in Table II lists the isosmotic values in moles of NaCl of the sera of these three species of fish. In general these isosmotic values are higher than the isotonic values obtained by the other methods. In the case of mackerel serum this difference is statistically significant. The reason for this hypertonicity of isosmotic solutions may be attributed in part to the difference in the activity of ions at the freezing point and at room temperature. Of more importance is the response of the cell to a solution containing a single species of cation. In such a solution the cell surface may be modified or the rate of water penetration may be different from that in plasma. Either of these conditions could account for this hypertonicity.

Table III shows the actual freezing point depressions of the bloods of the fish in Table II. The percentage of cells of the teleosts was determined in defibrinated blood obtained from cutting the gills. These are somewhat lower than values found for blood obtained from heart puncture. The pH values are for aerated blood and quite similar to the pH of the blood of other vertebrates.

The data in Tables IV and V were obtained using the densimeter method for the determination of isotonicity. The accuracy of this method is ± 0.001 *M* of NaCl and the maximum variation (for blood from the heart or caudal vessels) among fish of the same species was ± 0.01 *M* NaCl.

TABLE III

Physical data on fish bloods

Kind of fish	No. sampled	No. of measurements	pH		% cells	Freezing point ° C.
			Average	Range		
Mackerel	57	8	7.40	7.31–7.57	21.2±0.4*	−1.13
Tautog	17	5	7.27	7.08–7.48	20.5±0.8	−1.05
Dogfish	14	7	7.50	7.35–7.83	20.6±0.07	−1.95

* Standard error.

Table IV shows the dependence of the isotonic value upon the method of obtaining the sample of blood. The results with heart blood are thought to be closer to the true tonicity since the gill blood is probably contaminated with sea water and body fluids. Very striking is the fact that for either heart or gill blood there is a large variation in values between the various species of teleosts. The range is 0.10 *M* NaCl for the shark sucker to 0.26 *M* NaCl for the mackerel. These data necessitate a revision of the apparently over-generalized concept that the isotonic values for the teleosts fluctuate slightly around 0.25 *M* in NaCl, and also of our ideas concerning water balance in these forms.

Table V lists the isotonic values obtained for different elasmobranch bloods *after* osmotic equilibrium was established between the cells and the NaCl solution. In addition certain solutions listed in the "Formulae and Methods III" of the Marine

TABLE IV

Molar values of isotonic solutions for teleost red cells

Name of fish	No. studied	No. detn.	Average isotonicity Moles of NaCl	
			Blood drawn from	
			Gills	Heart
Shark sucker (*Echeneis naucrates*)	2	2	—	0.10
Weak fish (*Cynoscion regalis*)	1	1	—	0.13
Flounder (*Paralichthys dentatus*)	10	5*	0.16	0.14
Toadfish (*Opsanus tau*)	2	2	0.19	0.14
Eel (*Anguilla rostrata*)	1	1	—	0.15
Butter fish (*Poronotus tricanthus*)	4	2*	—	0.16
Sea robin (*Prionotus carolinus*)	5	4*	0.18	0.16
Menhaden (*Brevoortia tyrannus*)	19	8*	0.19	0.18
Sea bass (*Centropristus striatus*)	2	2	—	0.19
Porgy (*Stenotomus chrysops*)	8	3*	—	0.19
King mackerel (*Scombermorus cavalla*)	5	2*	0.21	0.19
Blue fish (*Pomatomus saltatrix*)	6	4*	0.25	0.20
Tautog (*Tautoga onitis*)	23	8*	0.27	0.21
Mackerel (*Scomber scombrus*)	65	10*	0.26	0.26

* Some measurements were made from pooled blood.

Table V

Molar values of isotonic solutions for elasmobranch red cells

Name of fish	Number studied	Isotonicity Molar NaCl	Elasmobranch soln.	Knowlton's soln.	Van't Hoff soln.	Lutz soln.
Smooth dogfish (*Mustelis canis*)	4	0.35	Hyper	Hypo	Hyper	Hypo
Sting ray (*Dasyatis centrura*)	2	0.33	Hyper	Hypo	Hyper	—
Skate (*Raja erinacea*)	1	0.34	Hyper	Hypo	Hyper	—
Sand shark (*Carcharias littoralis*)	1	0.35	—	—	—	—
			0.46*	0.41*	0.43*	

* Molar value of NaCl solutions of equivalent osmotic pressure as determined by ΔT_f measurements.

Biological Laboratory for use with elasmobranchs are evaluated. The value of the equivalence in moles of NaCl (obtained from the freezing point depression) of these solutions is also indicated. These data show that the range found in this group of fishes is from 0.33 M for the sting ray to 0.35 M NaCl for the sand shark and the dogfish. The range is very much less than that of the teleosts. The elasmobranch and Knowlton's solutions contain urea which explains the very large discrepancy between the evaluation of the isotonicity and the ΔT_f as discussed above.

CONCLUSIONS

The results require a closer appraisal of the physiological conditions for fish tissues suspended in artificial environments. This is particularly so for the teleosts since their bloods exhibit highly variable osmotic pressures. If the generally assumed value of 0.25 M NaCl were used, isotonicity would be approached only in the case of the mackerel. For the other teleosts examined, 0.25 M NaCl would be hypertonic and in some cases extremely so (as much as 250 per cent above normal for the shark sucker).

Although the elasmobranchs do not display the range in variation of isotonic values as do teleosts, it is important to realize that only two-thirds of the osmotic pressure of the blood is due to salts, urea being in large measure responsible for the remaining third. Thus, if 0.5 M NaCl were employed as a suspension medium, elasmobranch cells would be at a volume some 20 per cent lower than normal.

Emphasis should again be placed upon the very broad range of isotonic values found within the teleosts. This means that some teleosts have an osmotic pressure of their blood below that of some Amphibia. The indication is that the teleosts, considered as a group, have a more variable regulatory system than the higher groups of vertebrates as regards the solute concentration of the blood.

SUMMARY

1. Isotonic values of the blood of two groups of marine fishes were determined by three physiological methods. These were found to yield approximately the same results.

2. The freezing point depression was used to calculate isosmotic values of NaCl which proved to be generally hypertonic for the associated bloods.

3. The variation of isotonicity in the 14 teleosts examined ranged from 0.26 M NaCl for the mackerel to 0.10 M NaCl for the shark sucker.

4. Four different species of elasmobranchs were found to have isotonic values of the blood between the limits of 0.33 and 0.35 *M* NaCl. In addition, other commonly used artificial media were evaluated with respect to red cell isotonicity.

5. The extreme variability in the salt concentration of teleost blood, as determined by isotonic studies, emphasizes the need for further study of water regulation in this group of animals.

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THE FATE OF RADIOPHOSPHORUS INGESTED BY HABROBRACON FEMALES[1,2]

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In a previous publication the authors introduced the subject of the effects of phosphorus-32 on the development and heredity of offspring from adult insects given one feeding of the radioactive material (Grosch and Sullivan, 1952). The organism considered was the "ectoparasitic" wasp known in genetic literature as *Habrobracon juglandis* (Ashmead). Attention was directed to the curves representing daily egg production and to the hatchability of the eggs laid by samples of wasps after feedings at various levels of radioactivity. Subsequently we have attempted to clarify dosage relationships. In particular our desire has been to arrive at a basis for determining the dosage of radiation to which an egg may be subjected, and the present publication is concerned with some of the complications which need to be considered.

Two sources of beta radiation contribute to the dosage to which the egg contents are exposed: (1) the P^{32} in the soma of the female, and (2) the P^{32} herein shown to become incorporated into the egg. Therefore, in reference to (1), the present investigation of the consequences of ingestion of radiophosphorus was concerned with (a) observing change in the detectable radiation from wasps per unit time, an index of which is the biological half-life, and (b) illustrating changes in the distribution of radiophosphorus over the significant part of the ovulation period. Demonstrated in reference to (2) are (a) the channels whereby P^{32} may be lost by a female wasp and (b) the period during which a relatively high egg radioactivity obtains. Accordingly we are presenting the first study on insects which gives detailed chronological attention to excretory and ovipositional loss of radioactive substance subsequent to a single feeding of such material.

In addition to contributing toward a general understanding of what happens to ingested radioactive phosphorus, the present paper provides an explicit explanation of the upward slope of hatchability curves in the first week shown in the preceding report (Grosch and Sullivan, 1952). In reference to the 1952 report it should be mentioned that millicuries were erroneously indicated in the legends of several figures. Microcuries were intended throughout.

Materials and Methods

Virgin females from Habrobracon Stock No. 33 were fed mixtures of honey and P^{32} at various levels of radioactivity lower than the sterilization dose of 200 μc

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per gram of mixture. Each female was given one feeding only. Biological aspects of ingestion experiments and feeding technique have been treated in detail previously (Grosch and Sullivan, 1952).

After feeding, information on the distribution and excretion of radioactive phosphorus at desired intervals was obtained from the counting equipment of the Department of Experimental Radiology, Marine Biological Laboratory, Woods Hole, Massachusetts. In particular we wish to thank Mones Berman for helpful advice and direction. Counts were determined thrice in reproducible position for each specimen. Thus the starting point of analysis was a calculation of the average of three determinations.

Nearly 300 wasps were employed in obtaining daily measurements of radioactivity on the following: (1) whole animals, anaesthetized or dead, (2) separated parts, anterior and abdomen after transection at the petiole, (3) ovaries, a few dissections only, (4) eggs, in groups and as individuals, and (5) excreta. In following daily change in the radioactivity of the organism or in checking the radioactivity of a part or product, the wasps were handled in groups of various sizes as will be detailed below when presenting results.

The technique devised for investigating the radioactivity of excreta is new. It involves maintaining the female in a punctured No. 3 gelatin capsule with one host caterpillar (*Ephestia*). The puncture in the capsule wall seemed necessary to avoid oxygen depletion. Periodic transfer of the female is made at desired time intervals, one day in the present study, whereupon female and caterpillar with deposited eggs are removed from the capsule. The caterpillar is examined microscopically for purposes of transferring any excreta (by dissecting needle) from caterpillar to the capsule thus adding to the excreta deposited on the walls. The capsule is then digested in dilute HCl in a steel planchette. After dissolution the sample is evaporated and counted.

Radioautographs made in our laboratories by Mrs. Margaret T. MacLeod have proved helpful in interpreting distribution results and reference is made to them below. Standard procedures recommended by the Eastman Kodak Company were employed in making the autographs on their Nuclear Track Plates (beta) of the 1 × 3 in. size with 10 μ emulsion thickness.

RESULTS

Radioactivity of the organism

The time taken for the radioactivity of a wasp to fall to half of its initial value as the result of elimination and radioactive decay has been determined to be between four and five days as shown in Figures 1 and 2. This convenient index is known as the biological half-life. In addition, other details pertaining to change in radioactivity of a wasp can be discussed in reference to the figures.

Figure 1 presents comparatively the loss in radioactivity for three categories of whole animals: (1) killed immediately, (2) living but not ovipositing, and (3) living and ovipositing. Wasps of category (2) are infrequently seen and their ascribed lines are based only upon single animals. Also as shown by the ending of the pertinent lines, the life-span of non-laying wasps is short. The curves for non-layers are very useful in demonstrating the relatively slow loss of radioactivity when eggs are not being deposited. In categories (1) and (3) results for six

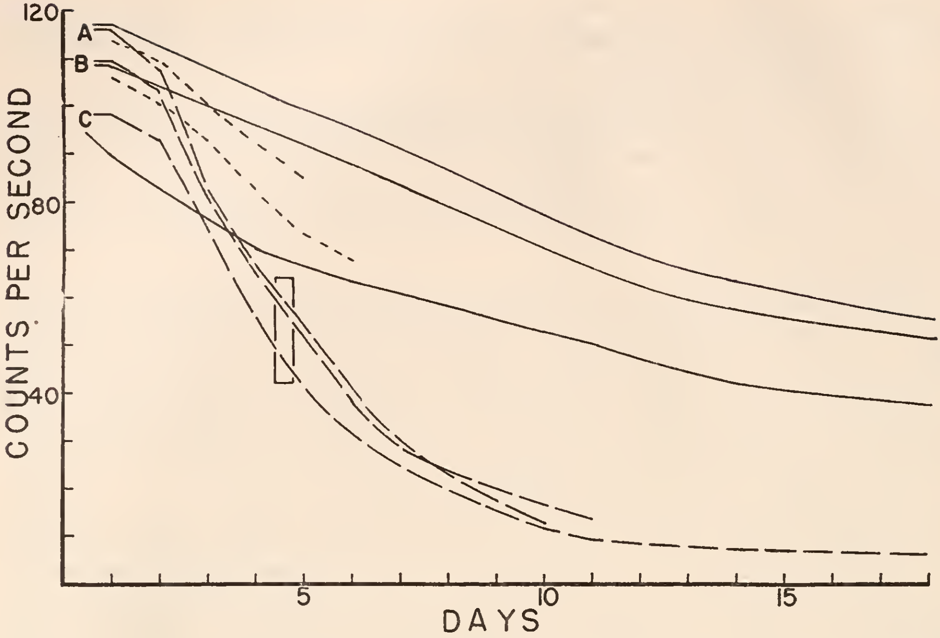

FIGURE 1. The radioactivity in mean counts per second when adult female Habrobracon are followed subsequent to ingestion of P^{32}. The solid lines are for wasps killed immediately after the single feeding. The short dashes are for live wasps not depositing eggs. The long dashes are for live wasps which are depositing eggs. The designation of the curves as A, B, and C on the basis of initial count is explained in the text. The rectangle drawn between the fourth and fifth day marks the time at which biological half life has been attained.

killed and nine ovipositing wasps, respectively, were obtained. The data from individuals in each category were grouped separately according to the level of radioactivity found in their initial counts:

Group	Initial count (c/sec.)	Individuals per group (1) Killed	(3) Ovipositing
A	110–119	2	3
B	100–109	2	3
C	90– 99	2	3
		—	—
		6	9

The six curves obtained by plotting the group means on successive days are shown in Figure 1. Since all females were allowed to feed once from the same honey-P^{32} mixture, the different levels of radioactivity found in initial counts are ascribed to chance variation.

To obtain merely a decay-plus-loss curve for ovipositing animals, all nine ovipositing animals could have been lumped and the mean value compared with that from a grouping of the six dead wasps. However such a procedure obscures the detail of the crossing of lines A and B shown between days 7 and 8. An explanation of this detail seems to lie in the number of eggs deposited. Whereas the wasps represented by line A had deposited an average cumulative total of 63 eggs per animal by the sixth day, those of line B had deposited only 61.6 eggs per animal. Line C which runs almost parallel to B represents females of quite similar egg productivity (59.9 eggs, cumulative total per animal). The implication of course is that the more eggs an animal lays per unit time, the more rapid may be its loss of radioactivity. The difference in slope between non-laying and laying live wasps points to the egg as a highly significant channel of P^{32} loss. Direct evidence of egg radioactivity is treated in the following section.

Figure 2 presents results based on larger numbers of animals in verification of the biological half-life. Thirty live ovipositing animals were followed for seven days. These animals comprised three groups which reflect the feeding of three

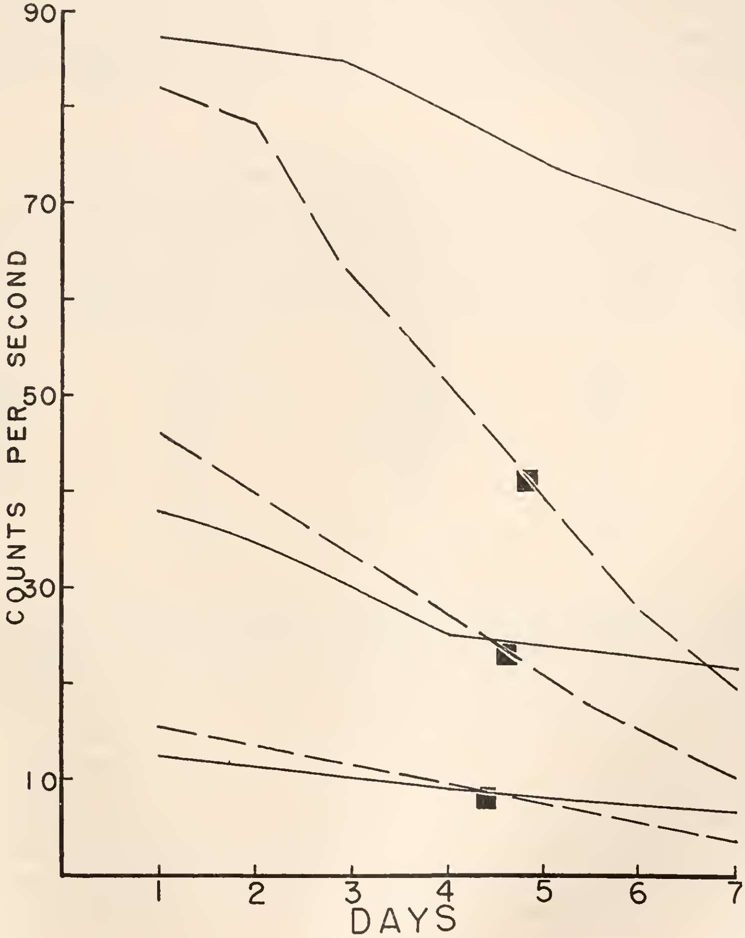

FIGURE 2. Verification of the results of Figure 1 using larger samples of wasps at three levels of radioactivity. The solid lines are for wasps killed immediately after feeding. They demonstrate measurable decay of P^{32} with a half-life extrapolating to between 14 and 15 days. The broken lines refer to egg-laying wasps with squares drawn to mark biological half life reached between four and five days.

different radioactive honey mixtures. Ten wasps were obtained therefrom with initial counts of less than 30 per second, 10 between 30 and 60 counts per second, and 10 between 60 and 100 counts per second. The group means of these live ovipositing wasps have been plotted as three broken lines to be compared with the solid lines which represent the mean values for 7 killed wasps numbering 2, 2 and 3 dead individuals per respective range of initial count. Here too it is demonstrated that the total radioactivity of an animal is related to the number of eggs deposited. By the fourth day the wasps counting less than 30 c/second had deposited an average total of 33.4 eggs per animal, those between 30 and 60 c/second had deposited an average total of 31.5 eggs per animal and the 60 to 100 c/second group had deposited an average total of only 24.2 eggs per animal. As indicated in Figure 2 the animals depositing the greatest number of eggs (wasps grouped by initial count of less than 30 per second) reached biological half-life first, while those depositing the fewest eggs (initial count more than 60 per second) reached biological half-life last.

Radioactivity of eggs

Figure 3 presents the results of following radioactivity for a week or more by taking GM counts of the eggs produced each day during that length of time. Depending upon the stage of her production cycle, a female deposits from 5 to 20 eggs per day. This number of eggs, whatever it totalled, was spread out on a planchette and counts per second were determined. For purposes of summary, the data which reflect feeding with four different honey-P^{32} mixtures, were grouped as follows:

Group	Number wasps per group	Initial count of wasps c/sec.	Total eggs per group	Max. level c/sec. per egg
A	9	above 90	393	10.99
B	19	60–90	1403	7.28
C	17	30–59	1133	5.32
D	5	5–15	819	2.09
Totals	50		3748	

The mean counts per egg for each group on successive days are plotted in Figure 3. A very definite relationship is thus shown between the radioactivity of females and of the eggs they deposit. Curve A reaches and continues at the highest values. Curves B, C, and D reach and continue at lower values in the order of the radioactivity of the wasps which deposited the eggs.

The curves for egg radioactivity are shown to have very typical shape. The level of activity rises sharply to a peak during the first two days, the maximum level obtained in each case. It then drops toward a relative low reached on the fifth or sixth day. In an additional experiment, not graphed, daily counts on eggs from a sample of 21 wasps were begun on the fourth day and made subsequently for a week, which proved tenable the supposition that the level of radioactivity of eggs continues to fall slowly. A total of 1075 eggs was considered by daily deposited groups in this experiment.

In further experiments, counts were made on single eggs of all those deposited by individual wasps on particular days subsequent to the ingestion of radioactive material. This was an attempt to obtain information on the variability in radioactivity of eggs within given days. Records of the radioactivity of 212 eggs were ob-

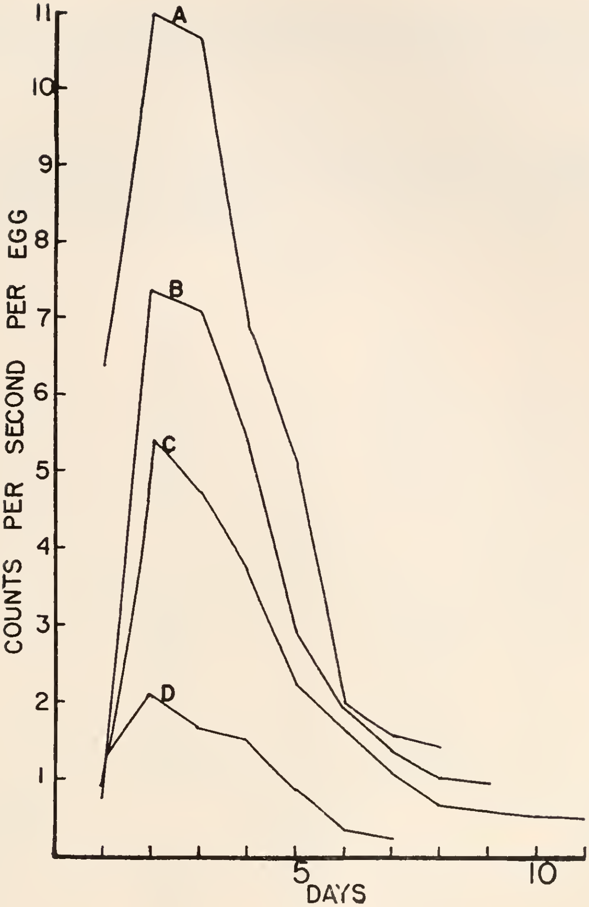

FIGURE 3. The radioactivity of Habrobracon eggs laid by samples of wasps after feeding once on honey containing P^{32} at zero days. Each line represents calculations of the arithmetic average for single eggs derived from GM counts per second on accumulations of eggs representing the daily production from individual wasps. The different level reached on day two reflects the initial level of radioactivity of the wasps depositing the eggs, and the designation A, B, C, and D is in order from high to low.

tained, representing conditions over the first five days of egg production. A variety of dosages were considered which resulted in wide differences in the means. The mean itself was not of primary interest here but rather the degree of variation around the mean to be represented as a comparative or relative measure. Such a measure is furnished by the coefficient of variation. Summarization is given in Table I. It will be noted that the comparisons are made between samples of approximately the same size, hence it is unlikely that S.D.'s and C.V.'s are varying because of chance inclusion of extreme variates in unequal sized samples. On the first day there is appreciable variation in radioactivity as shown by standard errors which are relatively large when compared with the mean. This is expressed in consistently higher coefficients of variation than are seen in subsequent days. Data for second and third days show variability similar to each other. Because of the time-consuming aspects of this type of investigation the eggs from few wasps could be handled on the fourth and

TABLE I

Summary of radioactivity data for single eggs in counts per second

Number of eggs	Mean	Standard deviation	Coefficient of variation
Day 1			
5	3.97	1.57	39.5
7	8.41	3.06	36.3
7	2.68	0.92	34.3
5	6.49	2.17	33.4*
7	8.45	2.36	27.9
7	2.78	0.58	20.8
5	37.96	5.80	15.3
5	24.46	2.47	10.1
Day 2			
9	14.18	3.09	21.8*
6	10.13	1.33	13.1
6	10.13	1.16	11.5
8	11.42	1.07	9.3
8	11.41	1.03	9.0
11	12.57	1.04	8.2
12	12.46	1.01	8.1
5	14.40	0.75	5.2
Day 3			
9	11.39	2.99	26.2*
11	10.79	2.62	24.2
8	45.61	5.96	13.0
14	20.10	2.36	11.7
8	13.18	1.22	9.2
4	36.85	3.02	8.2
8	30.62	1.89	6.1
Day 4			
6	9.27	2.15	23.1
4	46.05	5.19	11.2
5	5.96	0.62	10.4*
3	10.91	0.66	6.0
8	6.33	0.26	4.1
Day 5			
6	5.69	0.62	10.9
6	6.53	0.27	4.1*

fifth day; too few to permit conclusions to be drawn. After the fifth day, individual eggs count so very slowly that investigation of the radioactivity of single eggs had to be discontinued.

Admittedly it would have been ideal to have all records provided by the same animals followed for the whole period. However, in the absence of an automatic feeder device for the equipment used to measure radioactivity, it was humanly impossible to carry out the ideal design of experiment. Only in one case were the eggs from the same animal examined over the whole five day period. These are indicated on the Table by asterisks. Here there is suggested progressively less variation in successive days.

Radioactivity of excreta

The radioactivity of the excreta collected by the gelatin capsule method was measured daily for each wasp of a sample of twenty-five fed from the same honey-P^{32} mixture. Before and after the four-day duration of the experiment the radioactivity of each wasp was also measured. By correcting for isotope decay, the initial radioactivity of each wasp and of its excreta were put on a common basis with the final radioactivity of each wasp. The amount of loss was then calculated (by subtraction) for comparison with the total amount (addition) of radioactivity of the excreta. Table II presents a summary of these data for actively ovipositing wasps and gives the comparison as a percentage. Since it was the comparison which was particularly desired the data are organized with reference to percentage by a listing in

TABLE II

A comparison of the radioactivity of excreta with the total radioactivity lost in four days by ovipositing Habrobracon

Total radioactivity of excreta in c/sec. corrected for decay	Loss in radioactivity not due to decay c/sec.	Per cent of loss through excreta
10.18	48.22	21.1
5.81	25.11	23.1
10.78	44.63	24.2
9.47	36.95	25.6
15.67	55.76	27.6
19.69	62.75	31.4
9.59	29.51	32.5
5.94	17.30	34.4
11.99	30.55	39.2
10.04	25.47	39.4
7.99	19.93	40.1
10.47	25.98	40.3
15.41	37.81	40.7
23.25	55.96	41.5
22.00	52.70	41.7
8.03	17.61	45.5
9.42	19.69	47.8
5.67	11.51	49.2
7.36	14.57	50.5
13.02	24.20	53.7
19.93	36.82	54.2
25.27	43.49	58.1
8.38	13.75	60.9

order from the lowest to the highest. A consideration of the distribution of percentages in Table II demonstrates that the mean, median and mode fall near 40%, a value which may be taken provisionally to represent the average loss of radioactive phosphorus to be expected to occur by way of the excreta for ovipositing females. Data for two females which laid few or no eggs have been omitted from the Table. Excreta accounted for 82% and 100% of their radioactive loss.

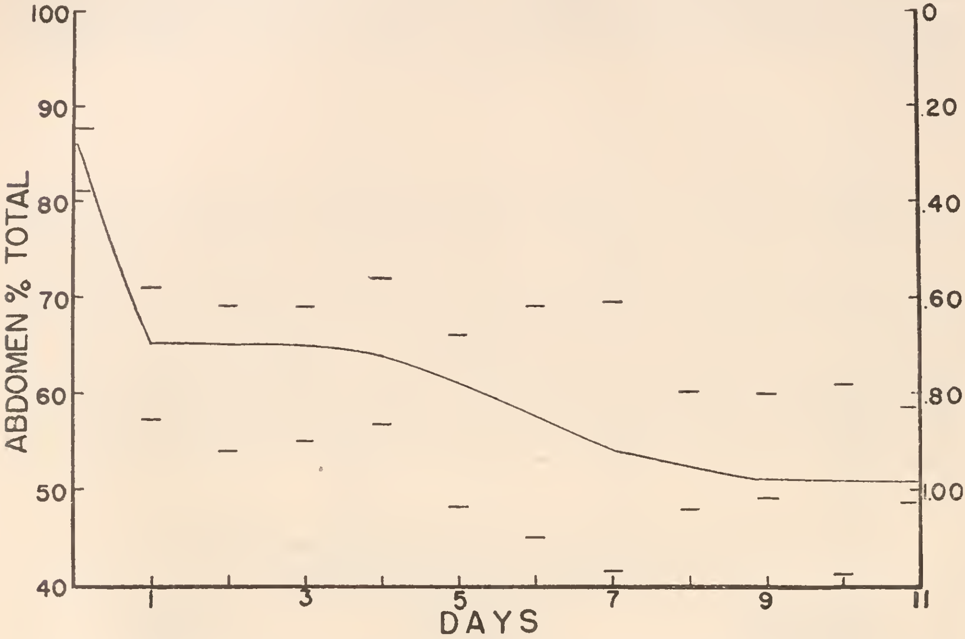

FIGURE 4. The distribution of P^{32} in Habrobracon females subsequent to a single feeding of the honey mixture. The mean value is plotted for the per cent of the total radioactivity demonstrable for the abdomen and the daily range is deliminated by dashes. Included on the right is a scale for interpreting the same results in terms of an anterior/posterior ratio.

Distribution of P^{32}

Transection experiments summarized in Figure 4 demonstrate that shortly after feeding, the abdomen contains a major amount (86%) of the radioactive material. This of course is to be expected since the crop, an organ which functions in immediate storage of food, has an abdominal location. Within twenty-four hours the proportionate distribution has changed so that only about 65% of the active material is abdominal. As shown, this situation remains constant during four successive days corresponding to the period during which the biological half-life is reached and during which appreciable amounts of radioactive material are egested and incorporated into deposited eggs. Subsequently, the abdominal radioactivity falls off so that from the seventh day forward there is only slightly more than 50% of the total radioactivity demonstrable as abdominal in a majority of animals. These conclusions are based upon transections of 134 females, of which 5 to 15 were sacrificed per day. Eggs laid each day were recorded and cumulative egg production for each female was calculated therefrom. Although oviposition has been shown above to be a very significant channel for exit of radioactive material and therefore a very important factor in the regulation of the total radioactivity of the animal, egg laying does not appear to be significant in determining P^{32} distribution.

Attempts to show a correlation between total eggs produced and proportionate distribution of P^{32} have failed. The evidence as given by scatter diagrams will not be presented. Instead the following selected examples will serve to demonstrate the point that although there may be appreciable differences in the number of eggs laid by females, however, provided an equivalent amount of time has elapsed since feeding, a very similar distribution of radioactive material may be expected:

Day	Abdominal per cent of radioactivity	Cumulative total eggs
7	53.67	94
	53.62	115
9	52.73	98
	52.82	56
11	52.23	183
	52.40	71

Apparently egg production is much less important in bringing about distribution than uninvestigated non-specified metabolic activities which appear to require elapsed time. Consistent with these conclusions is the fact that ovaries dissected out of adult females show very slow counts after biological half-life has been passed. In the present investigation, dissected ovaries were found to contain less than 33% of the abdominal radioactivity and one egg-laying female on the eighth day carried only 7% of her abdominal radioactivity in the ovaries.

The conclusion that physiological activities other than egg production are importantly involved in the distribution of radioactive phosphorus raises the question of where the material is present. The gonads apparently do not function as a storage depot but rather as disposal units which draw upon the reservoirs in the organism for the P^{32} to be incorporated into eggs. Thus distribution of radiophosphorus in Habrobracon is viewed as mobile and adjustable, an approach entirely acceptable on the basis of turnover studies in other organisms. One of the most definite of the general results emerging from tracer research is the demonstration of the dynamic character of molecules involved in metabolism (Kamen, 1947).

Radioautographs of Habrobracon females fed P^{32} are in accordance with these views in showing the trophocytes as the most radioactive elements of the gonads, while in addition other wasp tissues become particularly radioactive such as the muscles, the midgut epithelium, the brain and the central nervous system. Observations of radiophosphorus content of the tissues enumerated are consistent with reports on radioautographs of other insects (Lindsey and Craig, 1942; Irwin, Spinks and Arnason, 1950). Incidentally, because we are using P^{32} in amounts larger than tracer doses, the density of silver granules is great and resolution is lacking for some time after feeding. In fact, distinguishable details on wasp radioautographs were not clear unless the animal was sacrificed several days after feeding and our best radioautographs were made after biological half-life. The only detail discernibly distinct in radioautographs made one day after P^{32} feeding is the lack of radioactivity for the contents of the digestive tract in the region of the crop and stomach.

Discussion

A consistent picture emerges here of the loss of most of an ingested quantity of P^{32} within a few days after a single feeding of the radioactive material. Furthermore three lines of evidence point to the egg as a major channel whereby the isotope is eliminated from the fed female. In the first place, a comparison of curves in Figure 1 indicates the rapidity with which ovipositing wasps lose radioactivity in comparison to non-laying wasps. Secondly, the measurements of excretal radioactivity demonstrate that excretory deposits account for only around 40% of the loss in radioactivity. Finally, there is direct evidence from the demonstrated radioactivity of the eggs themselves. Thus an individual egg from a wasp fed P^{32} is subjected not only to the beta rays from the radioactive tissues and body fluids before laying, but irradiation is continued after deposit from the P^{32} incorporated into the egg.

Eggs laid the first day may be expected to have been in Metaphase I, a susceptible stage (Whiting, 1945), and hence influenced by maximum exposure to emanations from the soma of the mother. However, those laid subsequently up to about the sixth day were progressing through Prophase I, a relatively resistant stage, when the mother is most radioactive (Day 1). A great difference has been demonstrated between the eggs of day 2 and those of day 5 in the amount of egg-incorporated radiophosphorus. Curve A of Figure 3 shows that eggs on the second day after feeding can have as much as seven times the incorporated amount of radioactive material demonstrable a few days later. The period when incorporated P^{32} is at a high level is also when hatchability was found to be low particularly after a feeding of highly radioactive mixture (Grosch and Sullivan, 1952). Subsequently in the period when radioactivity falls off, the hatchability increases appreciably. We therefore believe now that egg radioactivity is most important in explaining hatchability during the 1- to 5-day period.

Eggs laid after the fifth day are not appreciably radioactive. Either initial exposure as mitotically active oogonia or total accrued dosage may be more significant in influencing their hatchability than the incorporated amount of radioisotope. In any event, it has become evident that eggs laid at different times by the same radiophosphorus-fed animal become exposed to quite different dosages of beta radiation from various sources.

The present authors are especially impressed with the concept of a gamete which carries built-in radiation equipment of particular potency in eggs laid early in the first week. Any susceptible stage in oogenesis or embryological development subsequent to incorporation may be subjected to ionizing radiations from a most intimate source, perhaps even from the chromosomes themselves.

There may be important genetic implications of the present results in an extension to what may occur in economically important vertebrates. For one thing, large amounts of radioactive phosphorus have been demonstrated to be removed from the physiological interior of the domestic fowl by way of the egg (Lorenz *et al.*, 1942). However attention has apparently not been directed to either the hatchability or the mutational potentialities of such radioactive products. Instead, interest has been devoted to applying tracer techniques to the physiological aspects of the treated adult rather than to investigating developmental or genetical consequences in the offspring (Entenman *et al.,* 1938; Hevesy, 1948; Lorenz *et al.*, 1942).

On the other hand, since there is no equivalent shell featuring inorganic constituents, it does not seem profitable to anticipate Habrobracon physiology on the basis of poultry work except in broad general terms. If the extension from vertebrate to invertebrate is valid, the element would soon make its appearance as organic phosphorus and as such be incorporated into eggs. The two days between feeding and the peak radioactivity of eggs seem to be an adequate period elapsed for synthesis.

SUMMARY

1. The biological half-life of radiophosphorus in egg-laying Habrobracon females is four to five days.

2. A majority of the P^{32} lost, about 60%, is by way of deposited eggs. This is based upon excretory studies using a newly devised technique, and upon comparisons of laying and non-laying wasps. Direct evidence of the radioactivity of the eggs is also presented.

3. The radioactivity of eggs rises to a peak reached the second day after feeding. Subsequent to the third day, it drops sharply to a relatively low plateau.

4. The variability in radioactivity from egg to egg is greatest in the first day. It may prove to be less each succeeding day.

5. During the first day almost 90% of the ingested radioactive material is abdominal due to the location of the crop. From the second to the fourth day about 65% of the radioactivity in the female is abdominal. Subsequently abdominal radioactivity approaches 50% of the total. Of these proportions less than one-third may be gonadal.

6. Although it is an important factor in the regulation of total radioactivity, egg laying has not been found significant in determining P^{32} distribution. A mobile, adjustable situation is indicated.

7. The shape of the hatchability curve previously reported for eggs from females fed P^{32} is interpreted for the first five days on the basis of gamete-incorporated radiophosphorus. Subsequently, initial exposure and accrued total dose may have greater significance.

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STUDIES ON THE INTEGUMENT OF THE SILKWORM, BOMBYX MORI. VII. THE PERMEABILITY OF THE INTEGUMENT TO OXYGEN AND CARBON DIOXIDE IN VIVO

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The fact that arthropod cuticle is permeable to gases, such as O_2 or CO_2, has been known in various materials (von Buddenbrock and von Rohr, 1922; Maloeuf, 1936; Fraenkel and Herford, 1938; and see the review by Richards, 1951). But in the silkworm any research on this respect is not found. The experiments reported here were undertaken to get quantitative data on the permeability to O_2 and CO_2 in the silkworm and, further, to know whether or not there is some difference in this property between the larva and the pupa.

On the other hand, that the insect cuticle, especially the epicuticle, is waterproof is a well known fact in general (Wigglesworth, 1948; Richards, 1951) and a procedure, abrasion of the cuticle with alumina dust, has been employed in the study of the transpiration of water through the cuticle. In the present research the effect of the same treatment on the permeability to O_2 and CO_2 was examined. Further, some other effects of rubbing are described here preliminarily.

MATERIALS AND METHODS

Two silkworm races, C122 × N122 and Taihei × Chôan, reared in the spring of 1952, were used as material. Oxygen uptake and CO_2 output were measured manometrically by Warburg apparatus with the materials operated as follows: (1) Normal larvae and pupae. The larvae were ligated immediately behind the head in order to avoid extrusion of the digestive fluid from the mouth, and at the level between the eighth and the ninth abdominal segment in order to prevent the excretion of excrements. (2) Sealed larvae and pupae. In the former all spiracles, and the parts of the body both anterior to the head ligation and posterior to the abdominal ligation were sealed with enamel paint, and in the latter all spiracles, the segments behind the seventh abdominal segment and the skin at the boundary between wings and body were sealed with it. Materials were placed in the vessels of the manometer 15 minutes after occlusion in the larva and 20 minutes after occlusion in the pupa. Readings were begun after a 15-minute equilibration period. Controls showed that the paint had lost its volatile components before measurements were made, and that neither the paint nor the ligation significantly affected the results. To measure CO_2 output, the method comparing O_2 uptake in the presence or absence of alkali was used. Besides 0.3 ml. of 10% KOH placed in the center well, 0.5 ml. of about 10% H_2SO_4 was placed in the side arm of the vessel for the purpose of taking up possible ammonia there. All experiments were carried out at 25° C. and the gas phase was air.

For certain experiments the surface of the cuticle was abraded by scattering alumina powder on hard paper and then rubbing the worm lightly against this repeatedly.

The time course of O_2 uptake did not show a linear relation but a slightly upward convex curve in the sealed worms, showing that the rate of O_2 uptake gradually decreased, as shown in Figure 1. This was also the case in CO_2 output. As it seemed possible to get more reliable and mutually comparable values in O_2 uptake and CO_2 output at the beginning of the measurement, μl. O_2 uptake or CO_2 output per gram live weight in the first 30 minutes was used as a measure of the amount of gas penetration through the skin. The number of sealed silkworms used varied from three to five for one vessel, and two to four measurements were repeated.

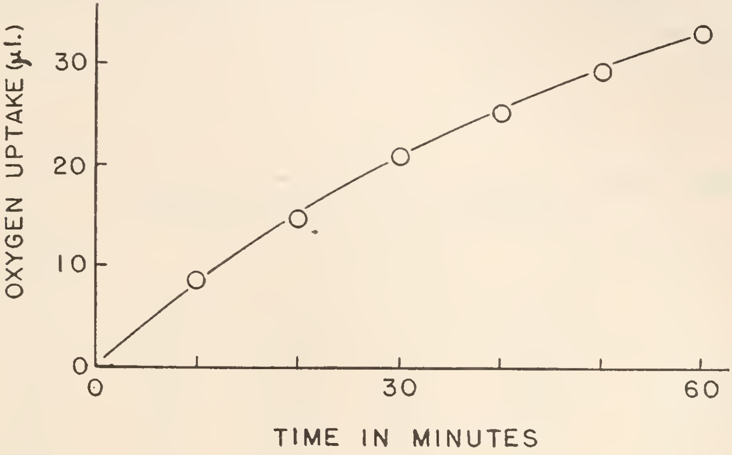

FIGURE 1. Time course of oxygen uptake of the closed silkworm larva. (μl./g. live weight).

It must be noted before the explanation of experimental results that there is no sure method of deciding whether the gasometric data obtained in the occlusion experiments are direct results of the gas penetration through the integument or not. But judging from the facts that the spiracles were perfectly occluded, that the film of the paint was considerably thick, and that there seems to be no part from which gases can penetrate out, it appears that the measured data in the occlusion experiments show practically the volume of gases penetrating through the integument.

Oxygen Uptake and Carbon Dioxide Output in the Sealed Silkworm

The results of the experiments with fifth instar larvae as well as the pupae are given in Table I. From the values obtained in the sealed silkworms, it is obvious that the integument is permeable to O_2 and CO_2 both in the larva and pupa *in vivo,* even though the amount of gases is very much reduced in the sealed ones, to about one-fiftieth of the normals. Especially, after the middle time of the pupal stage, O_2 can be hardly measured. It has been already shown in the silkworm that the rate of the respiration varies during development (Itaya, 1940). This is shown also to be valid for the normal silkworms in Table I, where the respiratory rates similar to those obtained by him are represented, but not for the sealed ones. In other words, the rate of O_2 uptake or CO_2 output through the integument is perhaps independent of the normal respiration. The values obtained in the sealed worms are relatively higher on the day of the larval and the pupal ecdysis, only, as compared with any other time. This may be caused by the fact that the cuticle is the thinnest at that time, both in the larva and pupa (Kuwana, 1933; Ito, 1951). However, such a phenomenon as the amount of gases passing through the integument being somewhat related to the thickness of the cuticle is not seen, after the first 24 hours of each ecdysis.

As shown in Table I, the values of the R. Q. are below 1.00 in the normal worms but considerably higher in the sealed ones (see also Table II), like those obtained in blowfly larvae by Fraenkel and Herford (1938). The R. Q. is distinctly different in the larva and the pupa, being 1.00 to 1.20 in the former and approximately 1.50 in the latter (one exception in the larva may be an experimental error).

TABLE I

Oxygen uptake, carbon dioxide output, and respiratory quotient in the normal and sealed silkworms (μl./30 minutes/g. live weight)

Stage	Normal silkworm			Sealed silkworm		
	O_2 uptake	CO_2 output	R. Q.	O_2 uptake	CO_2 output	R. Q.
1-day larva*	456	411	0.90	12.5	14.3	1.14
2-day larva	699	631	0.90	8.5	10.0	1.18
6-day larva	696	678	0.97	7.6	8.4	1.10
7-day larva	392	323	0.82	7.8	10.5	1.35
8-day larva†	428	360	0.84	9.2	9.2	1.00
10-day larva‡	241	205	0.85	9.4	10.0	1.06
1-day pupa§	190	168	0.88	12.2	19.1	1.57
4-day pupa	84	75	0.89	5.7	8.4	1.47
8-day pupa	179	165	0.92	±	11.2	—

* All larvae are in the 5th instar.

† The day of ripening (worms begin to spin cocoon).

‡ The third day after ripening.

§ Several hours after pupation.

Since the curve of the time course of O_2 uptake or CO_2 output in the sealed material is slightly convex, as shown above (Fig. 1), it is imagined that the rate of gas passage through the integument and the R. Q. value change according to the length of time after occlusion. The results of experiments in this connection are represented in Table II, in which the degree of change expressed by the ratio is also shown. In all cases the rate of O_2 uptake as well as of CO_2 output decreases and the ratio of decrease is more remarkable in the larva than in the pupa (see Fig. 2). The rate of O_2 uptake decreases less than that of CO_2 output and as a consequence the R. Q. becomes low both in the larva and pupa.[1] But several hours after pupation (when the quinone-tanning process is not yet finished) the decrease is found relatively remarkable and is similar to that of the larva.

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[1] In the first-day pupa, however, somewhat increased R. Q. is gained. This seems to be caused by the presence of difficulty in manometer experiments in the time when the respiratory rate is very much depressed, in some time, to the extent of a scarcely recognizable value on the manometer.

The rate of decrease shown in Table II is illustrated in Figure 2, in which all the data on the larval or pupal stage are collectively arranged according to the length of time after occlusion, as the degree of change in O_2 uptake or CO_2 output remains practically constant throughout larval or pupal stage. It is shown from this that the ratio decreases almost constantly according to the time after occlusion, regardless of stage. This tells us also that the amount of O_2 and CO_2 passing through the integument per gram live weight is nearly constant in the larva and pupa, respectively, except for the short period after the ecdysis, as mentioned above.

TABLE II

The change of oxygen uptake, carbon dioxide output, and respiratory quotient after occlusion of spiracles (μl./30 minutes/g. live weight)

Stage	Time in hours after occlusion*	Measured value			Ratio to the initial value (%)	
		O_2 uptake	CO_2 output	R. Q.	O_2 uptake	CO_2 output
2-day larva	0	8.5	10.0	1.18	100	100
	2	6.6	6.4	0.97	78	64
3-day larva	0	9.5	9.7	1.02	100	100
	3	4.2	3.6	0.86	44	37
5-day larva	0	11.8	12.3	1.04	100	100
	3	6.7	6.4	0.96	57	52
6-day larva	0	7.6	8.1	1.07	100	100
	1.5	6.4	5.9	0.92	84	73
1-day pupa	0	12.2	19.1	1.57	100	100
	2	8.4	14.8	1.76	59	77
4-day pupa	0	5.7	8.4	1.47	100	100
	2.5	5.2	6.7	1.29	91	80
8-day pupa	0	±	11.2	—	—	100
	3	—	9.0	—	—	80

* The second measurement of each stage was performed using the same worms.

MEASUREMENT IN THE RUBBED SILKWORM

The fact that the rate of transpiration of water is much increased when the most superficial layer of the insect cuticle is abraded by rubbing lightly with alumina dust is well established, but no information is available about the effect of rubbing on the permeability to gases. To know the effect of it in the silkworm, measurements were performed in the same way as in the preceding section, employing the worms rubbed with the powder immediately before spiracular sealing. The data are shown in Table III. From this it is obvious that when worms are rubbed, the rates of O_2 uptake and CO_2 output are increased as compared with

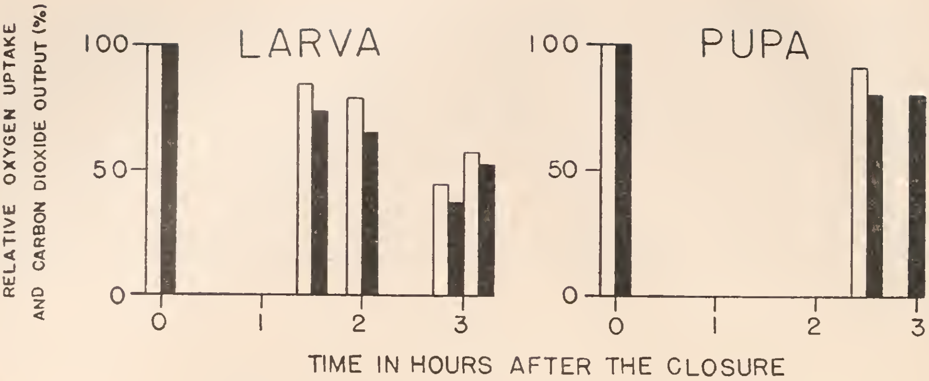

FIGURE 2. The decrease of oxygen uptake and carbon dioxide output of the sealed silkworm with the time lapse after closure (measurements for every 30 minutes). White column, oxygen uptake. Black column, carbon dioxide output.

the unrubbed worms (compare Table III with Table II). The increase of CO_2 output is proportionately one and a half times as high as the increase of O_2 uptake (Table IV). Further, the increase for both O_2 uptake and CO_2 output is a little higher in the larva than in the pupa, without exception. The fact that the increase of CO_2 output is higher than that of O_2 uptake throughout all experiments causes the value of the R. Q. to reach to a level as high as about 1.50 in the larva and more than 2.00 in the pupa.

The rate of O_2 uptake and CO_2 output decreases according to the time lapse after occlusion (Table III and Fig. 3), like the case mentioned above (Table II and Fig. 2). But the decrease of O_2 uptake is less marked than that of CO_2 output, especially in the larva. Therefore, reduction of the R.Q. value is remarkable in the larva.

TABLE III

Oxygen uptake, carbon dioxide output, and respiratory quotient of the silkworms rubbed on the cuticle with alumina dust (μl./30 minutes/g. live weight)

Stage	Time in hours after occlusion	Measured value			Ratio to the initial value (%)	
		O_2 uptake	CO_2 output	R. Q.	O_2 uptake	CO_2 output
3-day larva	0 3	25.6 19.4	36.1 18.9	1.42 0.97	100 76	100 52
5-day larva	0 3	21.5 19.0	34.2 16.9	1.59 0.89	100 88	100 49
1-day pupa	0 2	14.6 10.8	37.9 23.9	2.60 2.21	100 74	100 63
4-day pupa	0 2.5	8.1 7.2	17.9 13.3	2.21 1.85	100 89	100 74

TABLE IV

The ratio of increase of the rate of oxygen uptake as well as carbon dioxide output by abrasion in the sealed silkworms

Stage	O_2 uptake	CO_2 output
3-day larva	2.7	3.7
5-day larva	1.8	2.8
1-day pupa	1.2	2.0
4-day pupa	1.4	2.1

SOME REMARKS ON THE RUBBED SKIN

The cuticle begins to darken at the rubbed part a few hours after rubbing and becomes black or dark brown in color about 24 hours later. The color differs a little according to the different developmental stages and is similar to the color developed in the body fluid exposed to air. The darkening occurs both in the larva and pupa but there is some difference in the time before melanosis [2] according to the developmental stages. Another interesting fact observed in the rubbed integument is that the scales are not formed in the surviving adult, if the pupae are rubbed in the middle or later stage, though most of them die. However, if the rubbing is performed shortly before the larval or pupal ecdysis, the molted larva or pupa shows quite normal appearance. Histological observations will be described in the future.

DISCUSSION

From the occlusion experiment it is obvious that the integument of the larva or pupa in the silkworm is permeable to O_2 and CO_2 *in vivo*, at least in the sealed worms. There seems to be no direct way of deciding whether the cutaneous respiration reaches the observed value also in controls, but the opinion of Fraenkel and Herford (1938) that the amount of O_2 diffusing through the skin in the normal animals would be much less than in the closed animals, may be applied in the silk-

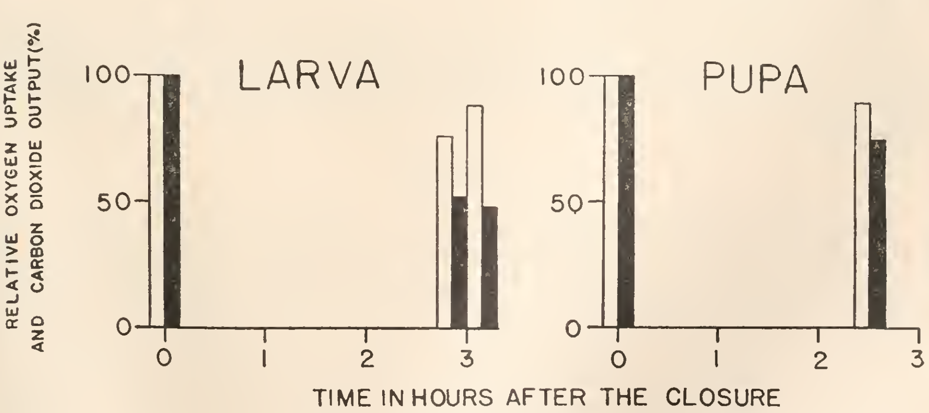

FIGURE 3. The decrease of oxygen uptake and carbon dioxide output of the rubbed and sealed silkworm with the time lapse after closure (measurements for every 30 minutes). The explanations are the same as for Figure 2.

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[2] The darkening of the cuticle is considered to be a kind of melanosis.

worm also. And judging from the very low rate of O_2 uptake or CO_2 output in the sealed silkworms, that is, about one-fiftieth of the normals, it is sure that the cutaneous respiration is so slight as to be practically negligible as compared with the tracheal respiration.

In the larvae of *Calliphora erythrocephala* (Diptera), *Chearocampa elpenor* (Lepidoptera), *Tenebrio molitor* (Coleoptera) and *Culex* sp. (Diptera), the rates of respiration in the closed ones range from one-half to one-fifth of the normal animals (Fraenkel and Herford, 1938), being much higher than the values obtained in the silkworm (about one-fiftieth). This difference may be attributed to the minute structure of the cuticle, blood physiology and so forth. But O_2 uptake calculated from Table I amounts, for instance,

to 0.025 μl./mg./hr. (one-day larva of the fifth instar), or
to 0.015 μl./mg./hr. (six-day larva of the fifth instar)

in the closed worm in air, and these values do not differ, in the order, as compared with the values shown in *Calliphora* by the above two investigators. It is supposed, therefore, that the low value, such as one-fiftieth of the normal in the sealed silkworms, would be brought about because tracheal respiration is far more important in this insect than in *Calliphora erythrocephala,* etc.

In the closure experiment of *Cambarus bartoni* it is shown that the rate of respiration becomes lower with the time lapse, and that the R. Q. value becomes higher in this course (Maloeuf, 1936). In contrast to this, it is shown that the R. Q. value is reduced in the sealed silkworm, though the same change as in the former animal is seen also in the latter one as for the time course of the respiratory rate.

It was shown in other experiments that the transpiration of water is much increased by rubbing in the silkworm pupa (Ito and Tanaka, 1952). In this paper it is shown that the rate of permeability to O_2 and CO_2 is increased by rubbing, but the ratio of increase in permeability to O_2 and CO_2 by abrasion is far lower than that to water by the same procedure.

The author wishes to thank Prof. A. G. Richards of the University of Minnesota for reading the manuscript and his kind aid in connection with its publication. Thanks are also due to Drs. T. Yokoyama and Z. Kuwana for reading the manuscript.

SUMMARY

1. From the results of measurements of O_2 uptake and CO_2 output in the sealed silkworm, it was confirmed that the gases penetrate through the integument both in the larva and pupa. The rate of O_2 uptake as well as CO_2 output is reduced to about one-fiftieth of the controls in the sealed silkworm. The R. Q. value of such silkworms ranged from 1.00 to 1.20 in the larva and was about 1.50 in the pupa. The rate of respiration became lower with the time lapse after occlusion, the decrease being more remarkable in the larva than in the pupa. Throughout the larval and pupal stages, the rate of O_2 uptake decreased less than that of CO_2 output.

2. The permeability of the skin rubbed just before occlusion increased a few times, the ratio being a little higher in the larva than in the pupa. The rate of in-

crease in CO_2 output was about one and one-half times that in O_2 uptake both in the larva and pupa. The decrease of the volume of measured gases seen with the time lapse in the rubbed material was not so remarkable in the pupa, and the rate of decrease in CO_2 output was relatively high in the larva. From this it was shown that the increase of cutaneous respiration by abrasion was not so remarkable as compared with that of water transpiration through the integument.

3. Some observations in relation to cuticle rubbing were briefly outlined.

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THE ORIGIN AND DISTRIBUTION OF NITROGEN IN TEREDO BARTSCHI CLAPP[1]

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The behavior and morphology of the Teredinidae dictate that the organisms obtain their nourishment either from the wood in which they live as adults, from particulate matter suspended in the water of the respiratory stream, or from a combination of these two sources.

Boynton and Miller (1927) demonstrated the existence of a cellulose-splitting enzyme in *Bankia setacea,* and Lane and Greenfield (1952) have reported the presence of a cellulase associated with the gut of *Teredo*. Previous papers from this laboratory (Lane, Posner and Greenfield, 1952; and Greenfield, 1952) have shown that in adult *T. pedicellata* glycogen may constitute up to 50% of the dry weight, and have suggested that this glycogen reserve could originate from the digestion of the cellulose matrix in which the marine borers live. These observations would appear to support the contention that *Teredo* can employ wood as food. On the contrary, it is a general lamellibranch habit to filter suspended food materials from the water of the respiratory stream, so it might be suspected that planktonic organisms would comprise the chief food supply of *Teredo*.

Greenfield (1953) has reported that the nitrogen content of adult Teredinidae is generally low, the average total nitrogen content of 179 animals being 1.77%. Thus far no work has been done to show the distribution of nitrogen in *Teredo*, nor to identify the raw materials out of which the proteins of the adult borer are constructed.

It is the purpose of this communication briefly to report studies designed to clarify the origin of tissue protein in the Teredinidae.

MATERIALS AND METHODS

Adult animals to be used for analysis were grown in pine collecting panels measuring 1″ × 6″ × 12″, immersed in Biscayne Bay in a locality known to be infected with shipworms. *Teredo* of useful size for analysis could generally be recovered between six weeks and two months after the original immersion of the wood. The collecting panels were usually completely destroyed by the end of six months.

All the larvae used in the present study were produced in the laboratory. Teredo-infested timbers were maintained in a laboratory tank of circulating sea water. Both supply and overflow lines were protected by 120-mesh Monel screen filters. At intervals of approximately 12 hours the larvae held on the efferent filter were washed into a container from which they could be recovered by filtration through paper, or by individual capture with a pipette.

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[1] Contribution No. 99 from the Marine Laboratory, University of Miami. These studies were aided by a contract between the Office of Naval Research and the University of Miami in cooperation with the U. S. Navy Bureau of Yards and Docks.

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Hydrolyses were effected with 6 N hydrochloric acid, 12 N sulfuric acid or with 10% (w/v) barium hydroxide. After appropriate neutralization and concentration the hydrolysates were chromatographed on Whatman No. 1 paper. Both one- and two-dimensional chromatograms were run to separate various components of the hydrolysates. Each ninhydrin-positive spot originating from the hydrolysates was identified by suitable standard solutions of known amino acids run simultaneously. Solvents employed were water-saturated phenol; the n-butanol, acetic acid, water mixture of Partridge (1948); and water-saturated collidine. Hydrolysis was shown to be incomplete at the end of twenty-four hours by the presence of unresolved peptides. Subsequently all hydrolyses were continued for 48 hours, at which time they were complete.

Wood for analysis was recovered from an uninfected portion of a regular collecting panel. The absence of infection was verified by X-ray examination.

Only complete, intact borers were used for analysis. The size ranged from 1.6 milligrams to 72.8 milligrams dry weight. These figures represent animals between approximately three and 150 millimeters length. Moisture content of adult *Teredo* of different sizes and at different times of the year has proved to average 73%. To determine whether amino acid content or composition differed with growth, the animals were divided into ten arbitrary weight groups with a range of from five to 10 milligrams per group. These groups were always separately analyzed.

The nannoplankton samples hydrolyzed for this study were obtained by filtering sea water through a No. 20 plankton net, discarding the organisms retained by the net, and passing the net-filtered water through an acid-washed asbestos filter pad. The filter and the retained materials were hydrolyzed *in toto*.

RESULTS

Preliminary comparison of acid and basic hydrolysates revealed no qualitative difference in the composition of the amino acid mixture resulting. Subsequent hydrolyses were always conducted in acid media.

The qualitative amino acid content of uninfected regions of wood is compared with the amino acids found in shipworms living in the same panels in Table I.

TABLE I

Qualitative amino acid content of Teredo and of organic components of its environment

Material hydrolyzed	L	PA	V	T	P	M	A	AR
12-hour larvae	X	0	X	0	X	X	X	X
Teredo less than 50 mg.*	X	X	X	X	X	X	X	X
Teredo more than 50 mg.	X	0	X	X	X	X	X	X
Pine wood	X	0	0	0	0	X	X	X
Nannoplankton	X	0	X	X	X	0	0	0

* Weights are dry-basis.

L—leucine; PA—phenylalanine; V—valine; T—tyrosine; P—proline; M—methionine; A—alanine; AR—arginine.

It will be observed that the wood is deficient in phenylalanine, valine, tyrosine and proline, all of which appear in the hydrolysates of adult shipworms. Hydrolysates of nannoplankton contain all the amino acids which are present in the shipworms but are missing from the wood, with the sole exception of phenylalanine.

The qualitative amino acid content of *Teredo* larvae is also shown in Table I. Larvae differ from the adults qualitatively only in the lack of the aromatic amino acids phenylalanine and tyrosine. It should be emphasized that the amino acids listed in Table I are the only ones found of more than 20 that were sought. Tryptophane, incidentally, has not been detected in any basic or acidic hydrolysate of shipworms of any size.

Discussion

The following experimental considerations are germane to the question of whether the wood excavated during the life of an adult borer—all of which must pass through the digestive system of the animal—could provide all of the nitrogen in the adult worm. The largest *T. bartschi* so far encountered in the exhaustive X-ray examination of more than 100 exposure panels measured 151.5 × 5.9 mm. The volume of this burrow, determined by filling it with paraffin and measuring the displacement of the wax, is most closely approximated by the cone formula. In this instance the maximum volume was 1380 $mm.^3$ This figure does not, however, represent the true volume of organic material in the borer. The mantle cavity is filled with water—often maintained under significant pressure (Lane and Tierney, 1951). This water is lost when the borer is removed from the burrow.

A series of fifty worms belonging to this largest size group was weighed. The average, drained weight was 0.350 g. with a moisture content of 73.0%. Total nitrogen (Kjeldahl) varied slightly about the figure of 1.75%. Thus, this largest example of *T. bartschi* contained 0.0016 g. nitrogen.

Wise (1946) states that mature white pine averages 0.3% nitrogen. If the moisture content be assumed to be 10% and the density to be 0.638 $g./cm.^3$, then the wood excavated during the life of this particular borer contained 0.0024 g. of nitrogen.

This would supply the nitrogen actually found in the animal (0.0016 g.) and leave an excess of only 0.0008 g. to supply that which must have been lost by digestive inefficiency, excretion and metabolism during the life of the animal. This may extend from ten to fifteen weeks. It is felt that this excess is inadequate quantitatively. The qualitative inadequacy of wood as a source of nitrogen for *Teredo* has been discussed above. On the contrary a safe excess of nitrogen in the form of amino acids shown to be deficient in wood, is available in nannoplanktonic proteins. It is suggested, therefore, that *Teredo* satisfies its nitrogen requirements by making use both of wood and of suspended planktonic organisms.

Summary and Conclusions

1. The amino acid content of hydrolyzed *Teredo bartschi* Clapp of all ages has been compared chromatographically with hydrolysates of pine wood and of nannoplankton.

2. Wood appears to be deficient in the aromatic amino acids phenylalanine and tyrosine, in the heterocyclic amino acid proline, and in valine, all of which are found in *Teredo*. Hydrolysates of nannoplankton contain all of these missing amino acids except phenylalanine.

3. It is suggested that both wood and suspended nannoplankton are used as dietary sources of nitrogen by *Teredo bartschi*.

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THE PHYSIOLOGY OF INSECT DIAPAUSE. VII. THE RESPIRATORY METABOLISM OF THE CECROPIA SILKWORM DURING DIAPAUSE AND DEVELOPMENT[1]

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The endocrine control of the pupal diapause of the Cecropia silkworm has been the principal theme of the preceding papers of this series. A considerable body of evidence is now at hand pointing to the prothoracic glands as the source of a hormone which reacts with the tissues to terminate the pupal diapause. The prothoracic gland hormone, in turn, has been identified as the "moulting hormone," the "pupation hormone," and the "growth and differentiation hormone" of previous investigators (*cf.* Williams, 1952).

The present paper initiates a series of reports in which the analysis is narrowed to the reaction of the hormone with the pupal tissues. The central though necessarily distant objective is to define the biochemical events which couple the action of the hormone to the biological end-results. It seemed possible that this elusive goal might be particularly accessible in the case of the diapausing insect where the prothoracic gland hormone produces prompt and spectacular effects in each and every tissue. As a first step in this direction the present study considers the respiratory metabolism of the Cecropia silkworm during diapause and development.

Materials and Methods

1. *Experimental animals*

The present investigation is based on approximately 1500 respiratory measurements performed on a total of 150 larvae, pupae, and developing adults of the giant silkworm *Platysamia cecropia*. One series of experiments was carried out on pupae of the closely related Cynthia silkworm (*Samia walkeri*). The insects were reared from eggs and managed according to techniques described previously (Williams, 1946).

2. *Apparatus*

Most of the measurements were performed at 25° C. in 45-cc. vessels equipped with venting-plugs and adapters for use with standard Warburg manometers (Fig. 1). In occasional experiments the gas volume of the system was decreased by the insertion of lucite "space-occupiers" into one or more vessels. Certain preliminary experiments were carried out in 25-cc. non-compensated capillary respirometers.

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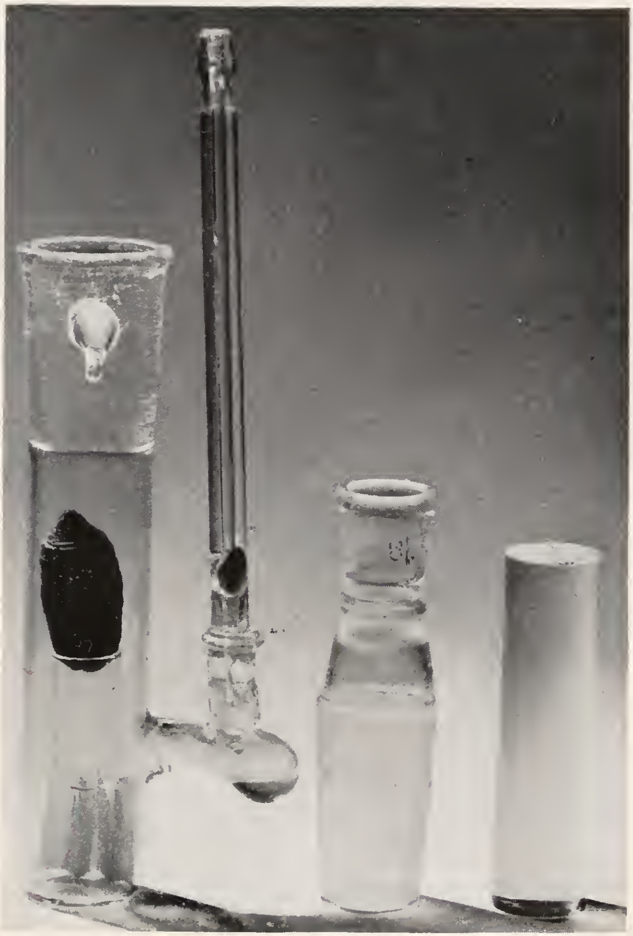

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FIGURE 1. Respirometer vessel for use with standard Warburg manometer. The vessel is equipped with a side-arm, a venting-plug, and an adapter for junction with the manometer. The pupa is supported on a glass tube above alkali-moistened filter paper. The lucite cylinder on the extreme right is a space occupier for increasing the sensitivity of the system.

The carbon dioxide output was absorbed on a strip of filter paper moistened with 0.3 to 0.5 cc. of 1.5 N sodium hydroxide. Measurements were performed over periods that averaged twenty hours, the excursions of the manometer being recorded at intervals of from five minutes to eight hours, as dictated by the rate of oxygen uptake.

At the conclusion of each experiment, acid was added to displace the carbon dioxide from the alkali, and the volume of the evolved gas determined in an analyzer of the type described by Bliss (1953). The several precautions outlined by Scholander *et al.* (1952) for experiments of this type were observed. The total output of carbon dioxide was determined from the moment the vessels were sealed until the end of the experiment, and the average calculated over this period. The over-all respiratory quotient of each insect was calculated by dividing the average rate of carbon dioxide output by the average rate of oxygen consumption.

Oxygen Consumption During Metamorphosis

Enormous changes were observed in the rate of oxygen consumption of the Cecropia silkworm during the course of its metamorphosis at 25° C. In Figure 2 these changes have been assembled and plotted, the discontinuity in the x-axis corresponding to the several months of temporary storage at 5° C. prior to the

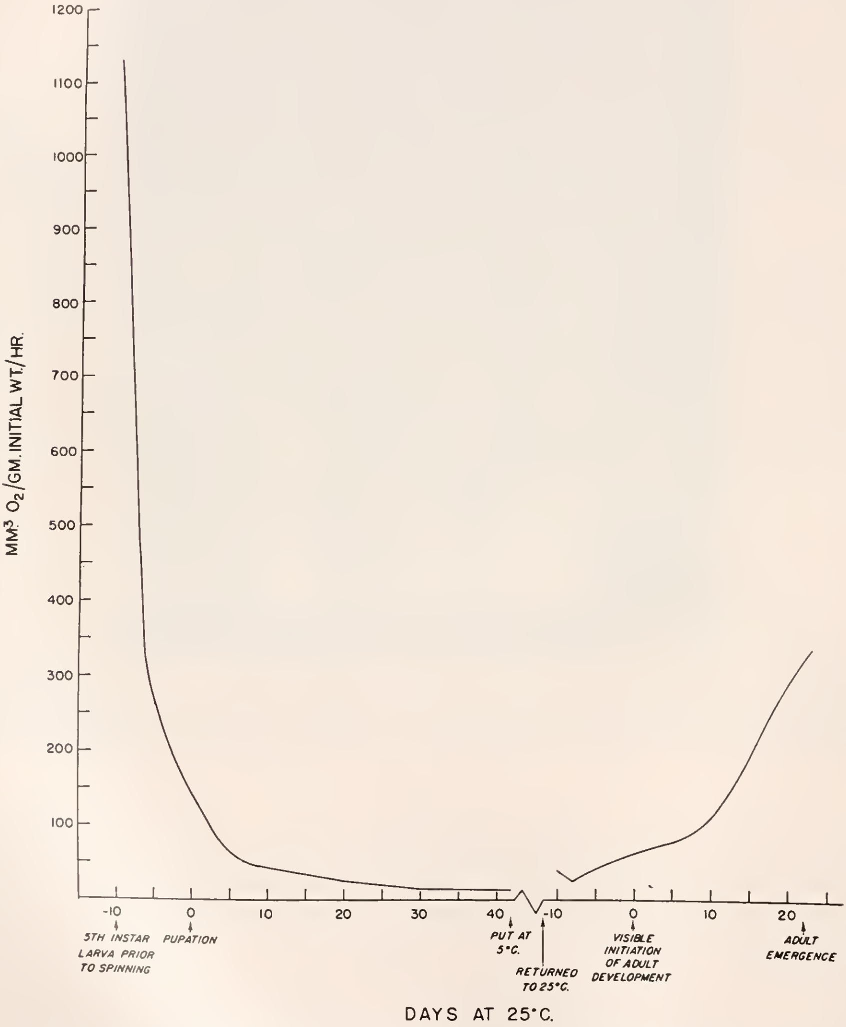

Figure 2. The average rate of oxygen consumption at 25° C. of the Cecropia silkworm during its life history. The rates of oxygen consumption are expressed as mm^3. oxygen per gram initial weight per hour; the "initial weight" refers to the weight of each individual two to three weeks after pupation.

return of the diapausing pupae to 25° C. In each instance the rates of oxygen consumption of the various animals have been computed in terms of the live weight of each individual several weeks after pupation. This procedure, the use of the pupal weights in the computations, automatically compensated for the large weight loss of the mature larva prior to pupation and of the pupa during the course of adult development.

The several segments of the curve will now be considered in further detail.

1. Larva, prepupa, and pupa

Figure 3 illustrates the precipitous decrease in the rate of oxygen consumption of Cecropia during the larval-pupal transformation. Similar measurements are recorded in Figure 4 for the closely related Cynthia silkworm. The animals were maintained at 25° C. and all measurements performed at this temperature.

It is clear that a striking decrease in the insect's metabolism begins just after the cocoon has been spun and continues throughout the prepupal and early pupal periods. By the second week after pupation the rate of oxygen consumption, in the case of Cecropia, is 2.5 per cent of that of the mature larva. By about the third week following pupation the rate of oxygen consumption reaches an extremely low level where it persists during the many months of pupal diapause at 25° C.

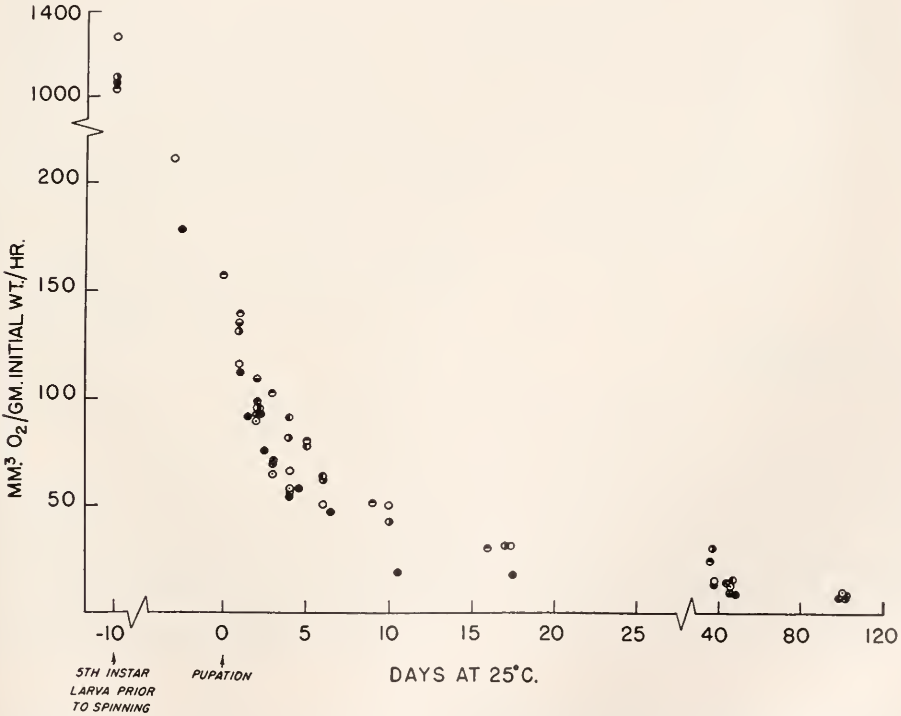

FIGURE 3. The rate of oxygen consumption of mature fifth instar Cecropia larvae at 25° C. just prior to spinning (ten days before pupation) and at frequent intervals thereafter. The measurements on larvae are determinations during periods when the animals were quiescent within the respirometers.

Ten to sixteen weeks after pupation, 43 diapausing Cecropia pupae, varying in weight from 4 to 6 gm., showed an average oxygen consumption of 16.3 ± 9.03 (s.d.)mm.^{3} per gram live weight per hour. The median was 13.7 and the extremes 6.8 to 38.0. On a dry weight basis the average was 65.0 mm.^{3} per gram per hour. These values are but 1.4 per cent of the corresponding measurements of mature larvae and 11 per cent of those of fresh pupae.

2. *Previously chilled pupae prior to the initiation of adult development*

Figure 5 records the respiration of five previously chilled pupae at frequent intervals after being returned to 25° C. The first visible signs of adult development (Williams, 1946) appeared after an average of 13 days at the high temperature. The behavior of these previously chilled diapausing pupae illustrates two significant facts—that the metabolism is still extremely low after prolonged exposure to low temperature, and that a progressive increase in metabolism becomes evident approximately a week prior to the first visible indication of adult development.

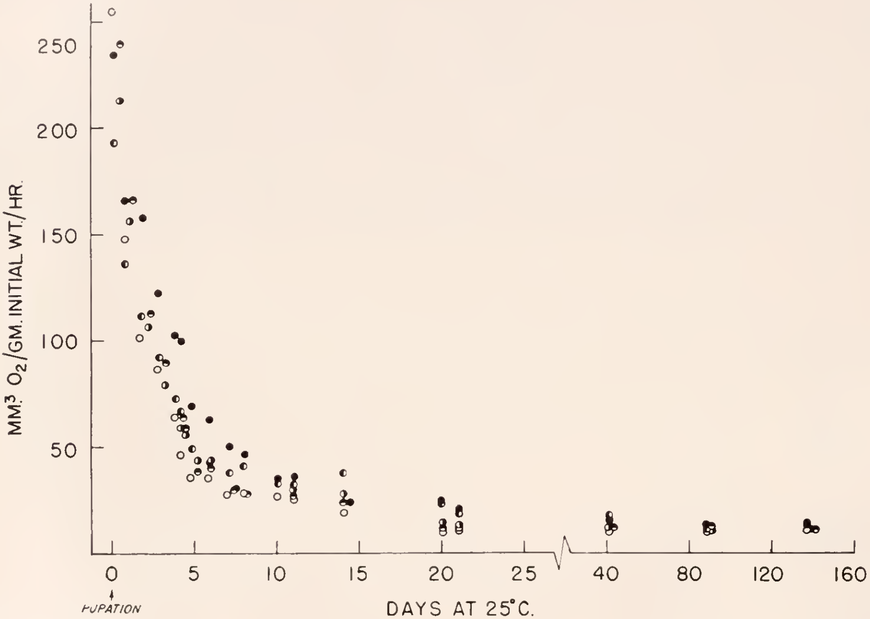

FIGURE 4. The rates of oxygen consumption at 25° C. of five Cynthia silkworms at the time of pupation and at frequent intervals thereafter.

Identical results were obtained in a second series of previously chilled pupae which initiated adult development after an average of 31 days at 25° C.

3. *After the initiation of adult development*

Figure 6 records the characteristic changes observed in the rate of oxygen consumption of several previously chilled pupae during the course of adult development at 25° C. The results are typical of a number of experiments of this type. The initiation of adult development is characterized by a continuation of the increasing metabolism which, as we have just seen, had already been evident a week before the visible initiation of development. During the final two-thirds of adult development, the rate of oxygen consumption increases yet more rapidly. Just prior to emergence, the average respiration of five fully formed adult moths was 312 mm.^{3} of oxygen per gram initial live pupal weight per hour. This value is 19 times that of diapausing pupae, but only ⅓ that of mature larvae.

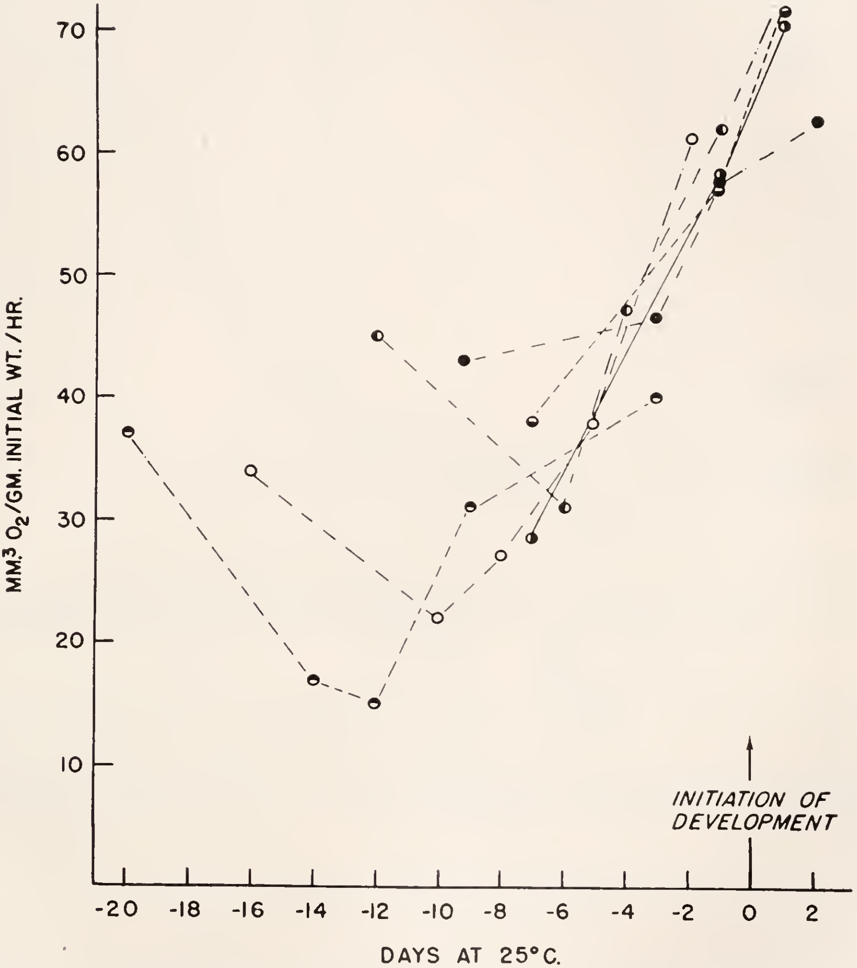

FIGURE 5. Rates of oxygen consumption of five previously chilled pupae after return to 25° C. The initial measurements for each animal were made two days after removal from the low temperature. The respiratory metabolism begins to increase about one week prior to the visible initiation of adult development.

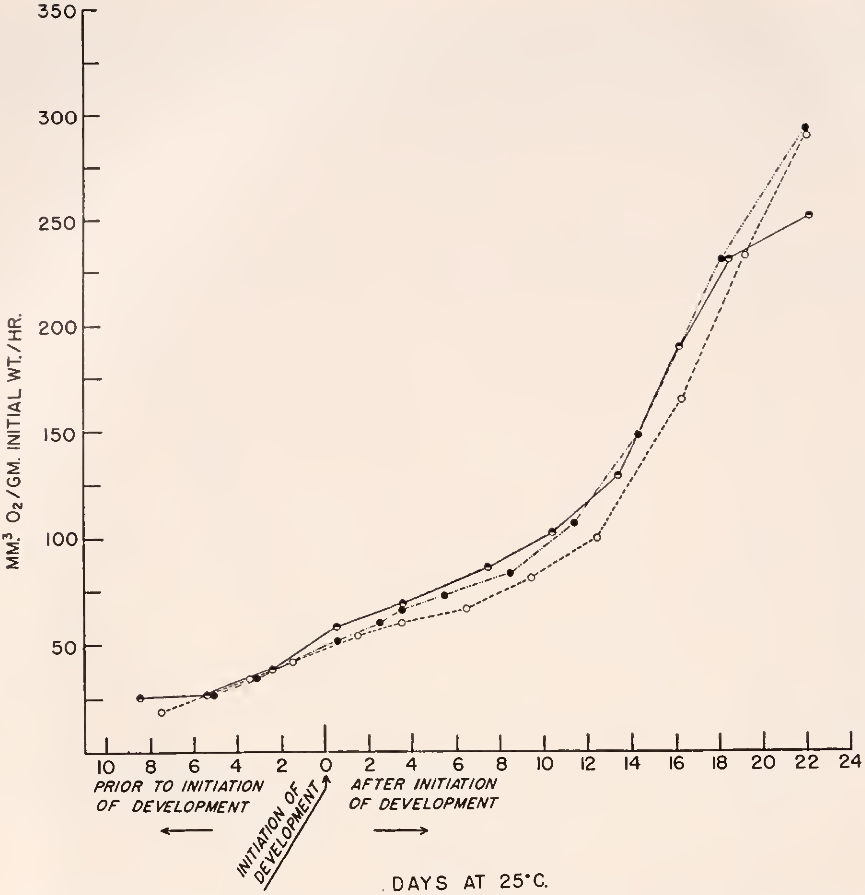

FIGURE 6. Rates of oxygen consumption during the course of the adult development of three previously chilled pupae. In this group of animals the adults emerged 24 days (average) after the initiation of adult development.

THE RESPIRATORY QUOTIENT DURING DIAPAUSE

1. Discontinuous release of carbon dioxide by diapausing pupae

The respiratory quotients of diapausing pupae were studied at 25° C. in animals previously stored for several months at this same temperature. In preliminary experiments an astonishing degree of scatter was encountered in the respiratory quotients when the latter were determined over one-hour intervals. Individual animals showed quotients which varied from 0.1 to 3.0 during successive hours—a result which suggested that either the intake of oxygen and/or the release of carbon dioxide were discontinuous.

Though a detailed study of the matter will be considered in a subsequent communication, we can state that experiments testing these possibilities have provided results of the type illustrated in Figure 7. Whereas the rate of oxygen utilization

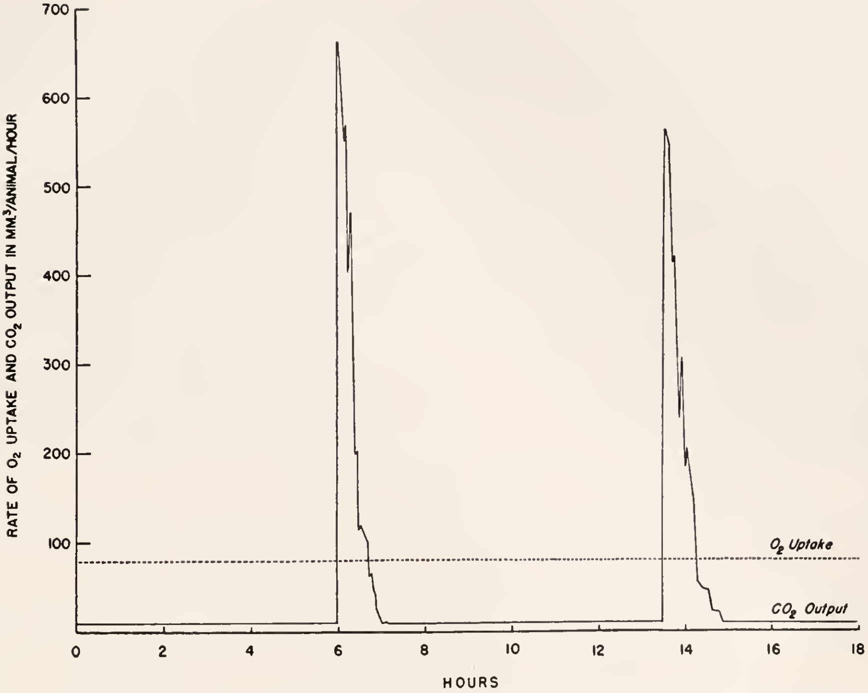

FIGURE 7. Rates of oxygen consumption and carbon dioxide output of a diapausing Cecropia pupa at 25° C. over an eighteen-hour interval. The "direct method" of Warburg was employed in this experiment.

was essentially constant, an extraordinary degree of discontinuity was observed in the rate of output of carbon dioxide. The latter was "blown off" in "bursts" every few hours, less than 10 per cent being excreted continuously.

We have consistently observed this phenomenon in all diapausing lepidopterous pupae examined—a finding in substantial agreement with the detailed results reported by Punt (1948, 1950). For our present purposes the discontinuous release of carbon dioxide is of interest because it illustrates a serious problem in the estimation of the respiratory quotients of diapausing pupae. It is clear that only prolonged measurements can provide a reasonable indication of the ratio between carbon dioxide release and oxygen consumption.

2. *Respiratory quotient of diapausing pupae*

The respiratory quotients of 49 diapausing Cecropia pupae were determined at 25° C. after three to five months of storage at this same temperature. Each experiment was continued for at least 17 hours in order to encompass two or more carbon dioxide bursts. The average respiratory quotient was 0.78 ± 0.093 (s.d.); the medium was 0.77 with extremes of 0.57 to 1.00. A similar series of determinations on seven brainless diapausing pupae recorded an average respiratory quotient of 0.72 ± 0.091 (s.d.); the median was 0.72 with extremes of 0.57 to 0.87. Consequently, there appears to be nothing peculiar about the average respiratory quotient of diapausing Cecropia pupae, provided that the experimental technique is adequate to cope with the discontinuous release of carbon dioxide.

STIMULATION OF PUPAL METABOLISM BY LOCALIZED INJURY

In experiments testing the metabolic effects of various drugs when injected into diapausing Cecropia pupae, a prompt increase in the rate of oxygen consumption was consistently observed. It soon became evident that the effect was due, not to the injected drug, but to the perforation of the integument in the course of the injection. For example, the same result was induced by puncturing the integument with a dry needle. The phenomenon was studied in further detail.

The effects of minor injury to the integument are illustrated in the data summarized in Table I. In this experiment the average rates of oxygen consumption and carbon dioxide output were first measured at 25° C. in a series of nine diapausing Cecropia pupae. A V-shaped incision having a total length of 4 mm. was then made in the thoracic tergum of each individual just lateral to the mid-line. Into three of the animals crystals of the anti-tyrosinase, phenylthiourea, were implanted at this time. The sites of injury were then sealed with melted paraffin. Twenty-four hour measurements of the rates of oxygen consumption and carbon dioxide output were then performed every few days.

TABLE I

Effects of small integumentary incisions on the respiration of diapausing Cecropia pupae at 25° C. The anti-tyrosinase, phenylthiourea, was implanted into three of the nine individuals at the time of incision (Group B)

Experimental time	Group A No PTU when incised (6 animals)		Group B Received PTU when incised (3 animals)		Over-all average (9 animals)	
	Relative rate of oxygen consumption	R. Q.	Relative rate of oxygen consumption	R. Q.	Relative rate of oxygen consumption	R. Q.
1 day prior to incision	100%	.81	100%	.88	100%	.84±.13 (s.d.)
1 day after incision	272%	.81	313%	.81	287±59 (s.d.) %	.81±.06 (s.d.)
6 days after incision	183%	.84	193%	.72	186±42 (s.d.) %	.80±.09 (s.d.)
22 days after incision	77%	—	81%	—	78±16 (s.d.) %	—

The rate of oxygen consumption of each individual began to increase within one hour and became maximal within twenty-four hours; at this time it averaged 287 ± 59 (s.d.) per cent of the pre-injury values (extremes of 224 and 424 per cent). Six days after the injury the rates of oxygen consumption were still elevated at 186 ± 42 (s.d.) per cent of the initial values. By the twenty-second day the injury metabolism had totally disappeared and the rate of oxygen consumption had returned to 78 ± 16 (s.d.) per cent of its value prior to injury.

Table I also indicates that the potent anti-tyrosinase, phenylthiourea, was without effect in opposing or preventing the injury metabolism. Moreover, since the respiratory quotient remained unchanged after injury, it is clear that the oxygen consumption and carbon dioxide production were stimulated in the same proportion.

The preceding experiments were performed on pupae capable of moving their abdominal segments. It seemed possible that the injury metabolism had its source in increased activity of the abdominal musculature. Two lines of experimentation were performed to test this possibility. In the first of these, the effects of injury were studied on isolated anterior and posterior ends of diapausing pupae. The injury metabolism was found to be just as pronounced in the immotile anterior fragments as in the motile posterior fragments. In the second type of experiment, a series of four pupae was immobilized by the removal of the entire chain of abdominal ganglia and connectives, thereby denervating the intersegmental muscles of the abdomen. Two months later the rates of oxygen consumption were measured, following which the thoracic tergum of each individual was punctured with a dry 22-gauge hypodermic needle inserted to a depth of 4 mm. Two hours after the injury the rates of oxygen consumption showed an average increase of 42 ± 17.5 (s.d.) per cent. It is clear that muscular activity is not responsible for the injury metabolism.

TABLE II

Effects of extensive injury to the integument on the respiration of nine diapausing Cecropia pupae at 25° C.*

Experimental time	Relative rate of oxygen consumption		
	Average	Median	Range
1 day prior to operation	100%	96%	69–145%
3 days after operation	814%	750%	570–1450%
69 days after operation	291%	207%	101–716%

* The integument overlying the pupal legs was removed and replaced by a triangular plastic slip sealed in place with melted paraffin. Crystals of phenylthiourea were implanted into each animal at the time of injury.

The magnitude and persistence of the injury metabolism appeared to vary with the extent of the injury. Major surgical procedures caused a tremendous increase in the rate of oxygen consumption which persisted for long periods thereafter. This fact is illustrated in the experiments summarized in Table II.

It is of particular interest and importance that diapausing pupae showed no over-all morphological change in response to the injury metabolism. Even when the metabolism was increased as high as 14-fold, the pupae continued to diapause—a fact confirmed by day-to-day observations and by dissections at the conclusion of the experiments.

DISCUSSION

Just prior to the spinning of the cocoon the mature fifth instar Cecropia larva ceases the intake of substances other than atmospheric oxygen. Henceforth until its death as a mature moth ten months later, the insect reworks according to its needs the materials of its own body. The larva, whose metabolism per unit mass ranks among the highest recorded for non-flying invertebrates, transforms into a diapausing pupa, whose metabolism is among the lowest noted for any animal (*cf.* Robbie, 1949; Heilbrunn, 1952). Then, months later, yet another transformation converts the diapausing pupa into the adult moth—a flying machine whose metabolism during flight is approximately two thousand times that of the pupa.

These results (Fig. 2) are in substantial agreement with those described by Heller (1926) for the diapausing sphingid, *Deilephila.* Like Heller, we observe that the months of pupal diapause are characterized by an extremely low metabolic rate, corresponding to the horizontal portion of the U-shaped curve of metabolism.

It is not our present purpose to consider the significance of the U-shaped curve in detail, but only to call attention to the metabolic characteristics of the two great periods of rapid rearrangement and maximal morphogenetic change. During the first of these, the prepupal period, the respiratory metabolism *decreases* more precipitously than at any other stage in the life history. During the second period of rapid change, the first few days of adult development, the over-all respiratory metabolism is extremely low—lower, in fact, than that which one can induce by injury to pupae during diapause. It is indeed true, as indicated in Figure 2, that the overall metabolism undergoes considerable increase during the latter stages of adult development. But this change mirrors the construction and maintenance of the future flying machine and, in our opinion, has no direct bearing on the earlier events which terminate the pupal diapause.

For these several reasons we are unimpressed by arguments which emphasize the high metabolic cost of morphogenesis and attribute the state of diapause to a simple quantitative deficiency in the over-all respiratory metabolism.

All of our experiments indicate that the oxygen consumption begins gradually to increase about one week prior to the visible initiation of adult development. Boell (1935) records a similar observation on diapausing grasshopper embryos where changes in respiratory rate were detected two days before the development of the embryo became apparent. This increment of respiration apparently signals a change in the pre-existing steady state of diapause and, in the case of Cecropia, suggests that the biochemical processes responsible for the initiation of adult development are set in motion about a week before one can detect any morphological evidence of the termination of diapause. The visible initiation of development is evidently the end-product of a brief period of endocrinological and biochemical preparation. Since the onset of adult development is brought to pass by the reaction between the growth and differentiation hormone and the diapausing tissues, the respiratory measurements suggest that the secretion of this hormone at 25° C. begins about a week before the actual initiation of adult development.

Turning to a consideration of the respiratory quotient, the measurements on diapausing Cecropia reveal nothing unusual, aside from the fact that the carbon dioxide is given off intermittently. A total of 49 determinations based on prolonged measurements showed an average respiratory quotient of 0.78 ± 0.093 (s.d.)—a value consistent with the combustion of a mixture of fat, protein, and carbohydrate. Though the respiratory quotients of diapausing lepidopterous pupae have hitherto been studied only by Agrell (1947, 1951a), the above-mentioned value is in good agreement with numerous determinations previously reported for non-diapausing pupae and for the changes occurring in the corresponding substrates (for summary see Needham, 1942, and Portier, 1949).

Our findings on Cecropia therefore stand in marked contrast to those described by Agrell (1947, 1951a) for the diapausing lepidopterous pupae, *Phalera* and *Endromis*. In measurements performed at 18° C. Agrell recorded respiratory quotients ranging from 0.1 to 0.3. On the basis of these extremely low values, Agrell has proposed a comprehensive theory of diapause (1951a, 1951b). According to this theory, the enzymes serving decarboxylation are defective in diapausing pupae due to a deficiency in thiamine and other vitamins acting as prosthetic groups in decarboxylases. The hormone which terminates diapause does so, he suggests, by promoting the synthesis of these particular vitamins, thereby reconstituting the decarboxylases. Previously thereto, the low respiratory quotients of diapausing pupae signal large scale conversion of fats into carbohydrates.

The respiratory quotients which formed the factual basis of this theory were determined by Agrell using the Thunberg microrespirometer. Each respiratory quotient was based on measurements over three to five successive periods of about one hour each. Agrell reports that the respiratory quotients obtained during these successive periods were uniform—a result indicative of a steady consumption of oxygen and release of carbon dioxide. But in our experience, as well as in that of Punt (1948, 1950), the carbon dioxide output of all the diapausing lepidopterous pupae examined (Sphingidae, Saturniidae, Noctuidae, Papilionidae) is characterized by singular discontinuity. In Cecropia at 25° C. less than 10 per cent of the carbon dioxide is released continuously. The remaining 90 + per cent is stored for periods averaging eight hours and then released within a one hour period (unpublished observations).

Though Agrell (1951a) suggests that the low respiratory quotients which he reports had (p. 283) "nothing to do with the rhythmic expulsion of carbon dioxide," it is difficult to be certain of this fact. Unless *Phalera* and *Endromis* differ from other genera and species of diapausing Lepidoptera, the release of carbon dioxide occurs during short, widely spaced intervals rarely encompassed within brief periods of measurement. In these same terms the increase in the respiratory quotient which Agrell observed at the outset of adult development could be accounted for, since the release of carbon dioxide is known to be less discontinuous after adult development has been initiated (Punt, 1948, 1950) and a representative sample could probably be obtained during brief measurements.

Since the determinations of Cecropia were performed over periods of not less than 17 hours, we conclude that the average respiratory quotient of 0.78 is a valid indication of the type of metabolism of diapausing Cecropia pupae, and that it probably signifies the combustion of a mixture of fats, proteins, and carbohydrates. Agrell's theory of diapause therefore appears to be unacceptable in the case of the Cecropia silkworm.

The injury-stimulated metabolism of Cecropia pupae is a phenomenon no less curious than the discontinuous release of carbon dioxide. A simple perforation of the integument reacts on the animal as a whole to cause a prompt and pronounced increase in the rates of oxygen consumption and carbon dioxide production. In the case of more extensive injury, the metabolism increases as much as 14-fold and requires longer than 2½ months to return to normal.

These results have certain parallels in the literature. Agrell (1951a) reports that the injection of physiological saline caused an 80 per cent increase in the respiration of diapausing pupae of *Endromis,* while Sussman (1952) has previously noted the phenomenon in the case of Cecropia. In *Calliphora* adults, injury is without effects on oxygen consumption (Thomsen, 1949) ; in *Periplaneta* adults, the injury-stimulated respiration persists for only 5 hours (Hassett, 1948) ; in the unfertilized eggs of *Bombyx,* only 24 hours (Wolsky, 1939). In this connection the large and prolonged changes in the colligative properties of the blood which Robinson (1928) observed after integumentary injury to diapausing pupae of the giant silkworm, *Telea polyphemus,* are of special interest.

The injury metabolism, as we have seen, is characterized by a proportional increase in both oxygen consumption and carbon dioxide output. The effect is not a by-product of increased muscular activity, since it persists in fragments devoid of somatic musculature and in animals in which the muscles have been denervated. Finally, the increased metabolism is insensitive to phenylthiourea and, consequently, is not mediated by tyrosinase.

Though the basis of the injury-stimulated respiration remains undefined, it is clear that the metabolism which it mirrors cannot be equated to the metabolism of the developing post-diapausing insect. For, though the rate of respiration of an injured diapausing pupa can increase to a level characteristic of animals halfway through adult development, no development occurs. Thus, the diapausing pupa, while capable of respiring at rates that characterize the growing insect, fails to grow in the absence of the proper hormonal stimulus and the proper *quality* of metabolism. It would appear that the increment of respiration induced by injury cannot be coupled to morphogenesis.

The injury-stimulated respiration, in itself, gives assurance that the absence of morphogenesis during diapause is not attributable to a simple *quantitative* deficiency in the dehydrogenase enzymes which release hydrogen from substrate, or in the redox enzymes which transmit the hydrogen to oxygen.

We wish to thank Mr. Dietrich Bodenstein and Dr. Leigh E. Chadwick for critical reading of the manuscript of the present paper.

SUMMARY

1. The over-all metabolism of the Cecropia silkworm, as signaled by its respiratory exchange, undergoes pronounced alterations during the course of the larval-pupal-adult transformation.

2. The rate of oxygen consumption of the diapausing pupa, averaging at 25° C. 16.3 ± 9.03 (s.d.)mm.^{3} per gram live weight per hour, is only 1.4 per cent that of the mature larva and 5 per cent that of the adult moth just prior to ecdysis. The metabolism at 25° C. persists at or near this low level throughout the pupal diapause.

3. One week prior to the termination of diapause the metabolism begins gradually to increase. This change apparently accompanies the initiation of the endocrine events which culminate in the termination of the pupal diapause. By the first day of visible adult development the rate of oxygen consumption is 3½ times that of the diapausing pupa.

4. The continuation of adult development after the termination of diapause is accompanied by further increase in the rate of respiratory exchange—a change which mirrors the differentiation of the adult moth.

5. It was found that localized perforations or other injuries to the integument of diapausing pupae caused a pronounced increase in the insect's metabolism. The magnitude and persistence of the enhanced respiration varied with the extent of the initial injury. After extensive injury, the metabolism increased up to 14-fold and required over 2½ months to return to initial levels. The injury metabolism was unaffected by the anti-tyrosinase, phenylthiourea.

6. Though the metabolism of diapausing pupa could in this manner be increased to levels characteristic of the post-diapausing insect mid-way in adult development, no development took place. The significance of this finding is considered in relation to the biochemical basis of diapause.

7. It was found that whereas the utilization of oxygen by diapausing pupae is continuous, the release of carbon dioxide is discontinuous. Ninety per cent of the carbon dioxide is accumulated and "blown off" in short "bursts" which, at 25° C., occur about once every eight hours. This fact necessitated prolonged experiments for the determination of respiratory quotients.

8. In experiments averaging 20 hours in duration, 49 diapausing Cecropia pupae showed an average respiratory quotient of 0.78 ± 0.093 (s.d)—a value consistent with the combustion of a mixture of fats, carbohydrates, and proteins during diapause.

9. No indication was found of the extremely low average respiratory quotients, the multiple deficiencies in vitamins serving decarboxylation, or the conversion metabolism which Agrell has postulated as the biochemical basis of the pupal diapause.

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THE DISTRIBUTION OF THE POLYCHAETE NEANTHES LIGHTI IN THE SALINAS RIVER ESTUARY, CALIFORNIA, IN RELATION TO SALINITY, 1948–1952

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The estuarine nereids, with representatives widely distributed, offer excellent material for studies in comparative physiology and ecology. The present study is part of an attempt to work out the nature of the adaptation to brackish waters as seen in one species occupying ecologically distinctive habitats within a limited geographical area. *Neanthes lighti* furnishes favorable material for studies of the invasion of fresh waters by marine annelids, since it equals or exceeds the much-studied *Nereis diversicolor* in its ability to live and reproduce in waters of low salinity. Its viviparous mode of reproduction, described in an earlier report (Smith, 1950), has perhaps been of adaptive significance in this respect. The fact that the species appears to consist of self-fertilizing hermaphroditic individuals, living in widely separated rivers, may have favored the appearance of local races, reproductively and spatially isolated from each other. In the Salinas River estuary, some twelve miles north of Monterey, California, the species occurs under a wide range of conditions, and so furnishes material for a study of ecological limitations and adaptations which can form a background for, and give meaning to, studies upon its physiology. As a preliminary to more detailed studies upon the mechanism of osmotic regulation, the present report describes the physical and biotic conditions under which *N. lighti* occurs in the Salinas River. These findings in themselves depict extremely variable estuarine conditions, and may be useful in studies on other animals, and in amplifying our information on estuaries in general. An understanding of the history of salinity changes in an estuary is a prerequisite for studies on the distribution and physiology of its inhabitants.

Observations were begun in 1948, coincidentally with other work, and have been carried out as opportunity offered until the fall of 1952. During certain periods the river could be visited only infrequently, hence the data include numerous gaps. However, the total four and one half year record does reveal in a general way the pattern of salinity changes in an estuarine system, and covers the full range of climatic conditions characteristic of this area. It is believed that the results clarify the relationship of salinity to climatic conditions, and will make future studies of estuaries in this area more rewarding.

Physical and Biotic Conditions in the Salinas Estuary

The estuary of the Salinas River (Fig. 1) presents an extremely varied set of conditions, especially in relation to salinity. This variation in salinity characterizes estuaries in general; for an excellent discussion of estuarine conditions, the review by Day (1951) should be consulted. The Salinas falls into Day's category of "blind" estuaries,[1] that is to say, it carries in most parts of the year so small a flow that across its mouth the sea builds up a barrier sand bar, blocking normal outflow and tidal exchange. When this has occurred, the estuary gradually freshens, often standing at a high level. In winter, flood conditions may occur following periods of heavy rain. In most years these seasonal freshets have caused the river to cut through the bar across its mouth, occasionally in the past rather violently, and with loss of life (MacGinitie, 1935), providing for a time an outlet to the sea. In recent years it has been the practice to excavate a channel through the barrier of sand in order to release the swollen river before flooding of farmlands oc-

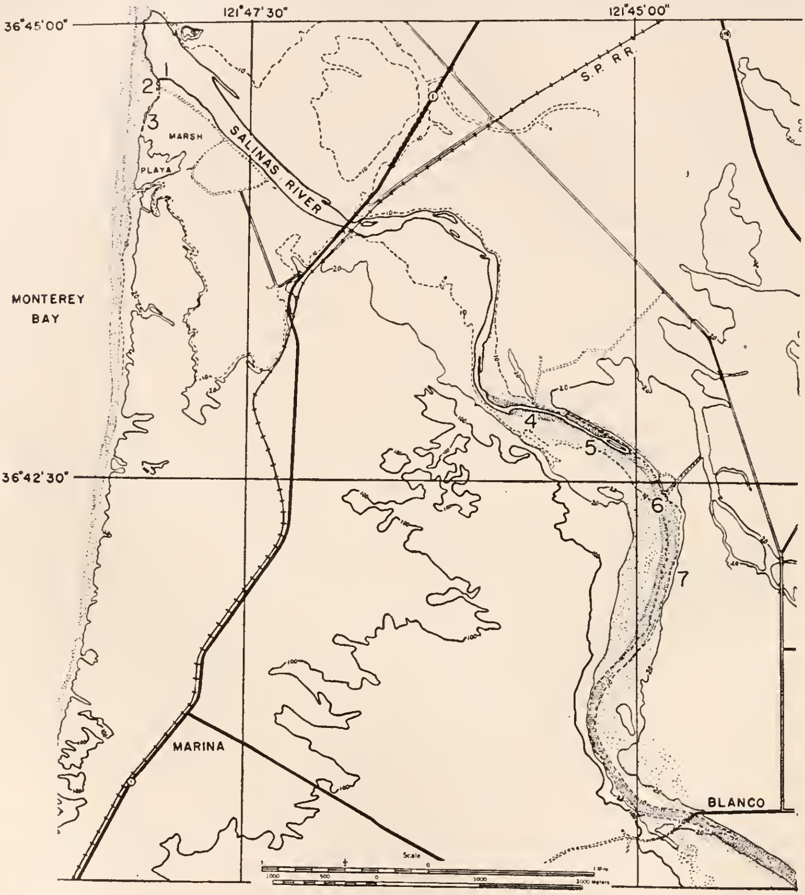

FIGURE 1. The estuary of the Salinas River, from Army Map Service sheets (Marina and Salinas quadrangles) compiled in 1947, to show locations 1–7 mentioned in text. Most of detail omitted; only 10, 20 and 100 foot contours shown.

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[1] Judging from the account given by Scott, Harrison and Macnae (1952), the Klein River Estuary in South Africa bears a close general resemblance to the Salinas Estuary.

curs. With the breaching of the barrier, the lower and middle reaches of the estuary receive tidal influxes of sea water until the bar is reëstablished. Thus the lower reaches for most of the year experience low salinity but are seasonally subjected to extreme fluctuations in salinity and exposure. The upper reaches of the estuary and marshes adjacent to the lower reaches also undergo salinity fluctuations, but not nearly so drastic in magnitude and rate of change. Were seasonal rainfall and the consequent fresh water discharge into the estuary uniform from year to year, a description of salinity conditions would be relatively simple, but in fact no such uniformity exists. In order to provide a background for understanding the changes in the river during the years 1948–52 of this study, rainfall and runoff records since 1944 have been compiled from published records (U. S. Depts. of the Interior and of Commerce). As shown in Figure 2, practically all of the rainfall in this region falls between September and April, and even within this span of months may be extremely concentrated, sending great freshets down into the estuary in certain winter months. The watershed of the Salinas is stated to be 4231 square miles, and rainfall varies considerably in different parts of the area. In order to make a rough estimate of the rainfall, the monthly rainfalls in inches reported from all stations on the watershed (4 to 8 stations in most months) were averaged, and the sum of the monthly rainfalls taken as an expression of total rainfall for each water year (October through the following September). These estimates showed a more than two-fold variation in annual rainfall, from about 10 inches in the dry years 1946–48, to 20 inches in the extremely wet 1951–52 season (Table I).

TABLE I

Total runoff in "second-foot-days"[2] entering Salinas Estuary by water-years (Oct.–Sept.), and totaled average rainfall at stations recording on Salinas River watershed

Water-year	Rainfall (inches)	Runoff
1944–45	12.11	157,147
1945–46	12.41	66,692
1946–47	9.25	3,513
1947–48	11.24	1,645
1948–49	11.56	25,500
1949–50	12.35	14,874
1950–51	10.21	17,862
1951–52	20.53	336,957

Discharge into the estuary has been taken from the records of the gauging station at Spreckels, a few miles upstream, near the city of Salinas. These values show a far more extreme variation than do rainfall records, the total yearly discharge varying from 1645 second-foot-days[2] (142,128,000 cu. ft.) in 1947–48 to 336,957 second-foot-days (29,113,084,800 cu. ft.) in 1951–52 (Table I and Fig. 2). Impoundments and irrigation diversions undoubtedly greatly reduced the discharge in the years of low rainfall, and may have contributed materially to the stability which characterized the estuary prior to 1949. Variation from month to month is also extreme, the record of 173,901 second-foot-days in the month of February, 1952 exceeding the combined discharge of the six preceding water-years.

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[2] One "second-foot-day" is the amount of water flowing in one day past a point at a rate of one cubic foot per second. It equals 86,400 cubic feet or 1.983 acre-feet.

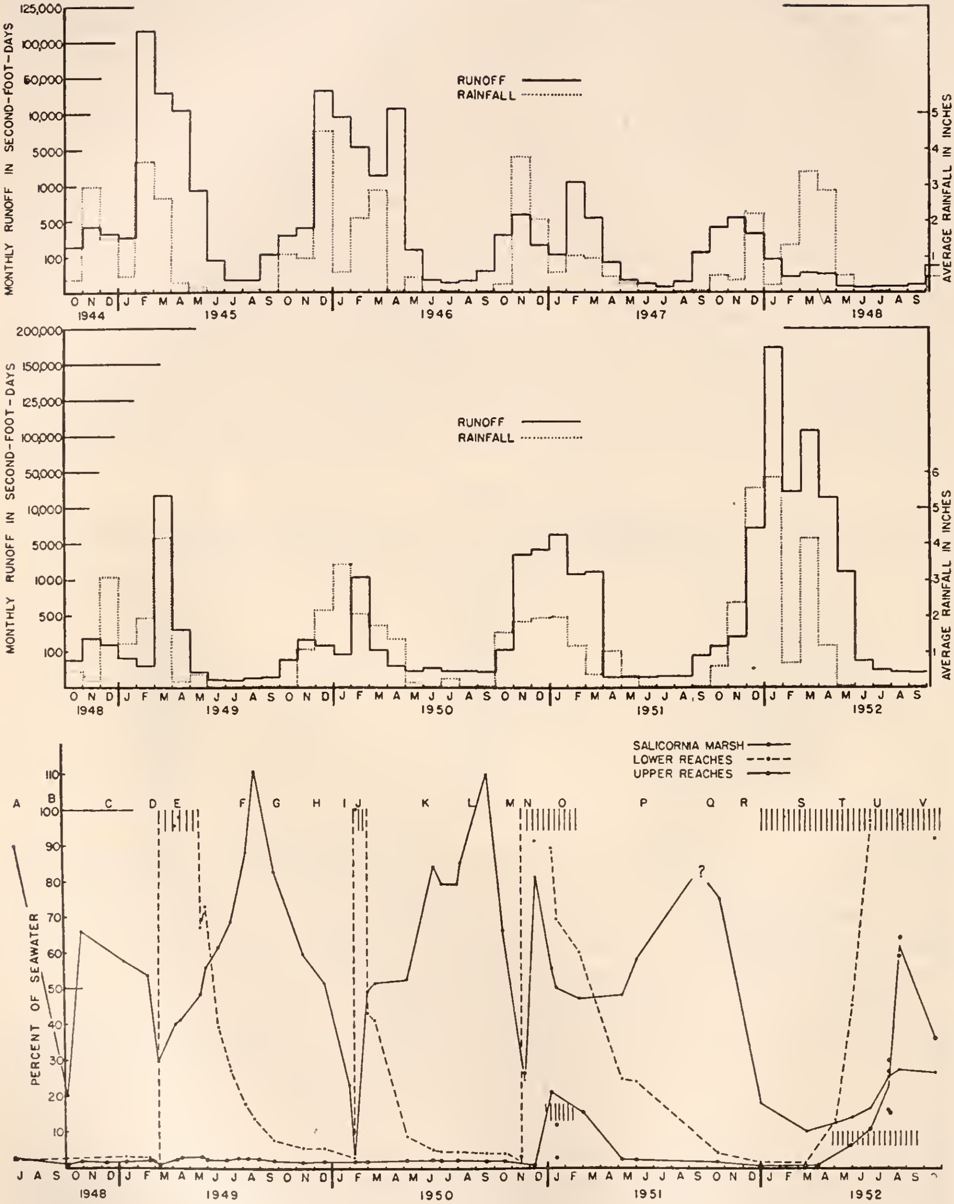

FIGURE 2. Upper and middle charts: monthly rainfall on Salinas River watershed and discharge into estuary at Spreckels, 1944–52 (note compression of runoff scale). Lower chart: salinity changes at Locations 3 (*Salicornia* marsh), 4 (upper reaches), and 1 (lower reaches), 1948–52. Letters above curves refer to points discussed in text.

Pattern of Annual Salinity Variation

It is apparent that an estuary such as that of the Salinas, in a region of variable rainfall, subject to summer diversion for irrigation and to winter floods, offers a most unstable environment, and one which can be described only in rather general terms. The observations reported below are admittedly rather superficial. The salinities are for simplicity expressed as "per cent of sea water" (taken as having a chlorinity of 19.64‰, or a salinity of 35.48‰). Obviously, calcium and other salts present in the distinctly "hard" Salinas River water render the river somewhat less unsuitable than its low chlorinity might suggest. This has been shown experimentally, but for purposes of giving the general pattern of variation in the brackish estuary, only chlorinity determinations have been used. Observations have usually been made at intervals of some weeks, since the Salinas lies about 100 miles from Berkeley, and were incidental to other work. Nevertheless, even scattered observations over a four-year period may contribute to an understanding of the range of variation in salinity experienced by *Neanthes lighti* in different typical habitats, and furnish a basis for appreciating the development of osmotic tolerance in a marine invader of brackish waters.

It should be noted that in the two dry winters prior to the start of this study, rainfall had been insufficient to cause the river to break through to the sea, and in the summer of 1948 the estuary was, accordingly, a quiet pond-like area of low salinity, a situation which became increasingly less stable during the following four years. Salinity determinations have been made regularly at four stations (Locations 1 to 4), and additional observations at certain others. The first three of the following stations lie in or adjacent to the lower reaches of the estuary; the last four are in the upper reaches and the head of the estuary (Fig. 1).

Location 1 represents the lower reaches of the estuary proper, below the bridges, where at times of tidal influx the salinity changes may be maximal and abrupt, but where for most of an ordinary year the water is of moderately low salinity. The bottom is of firm, somewhat muddy, fine sand, in some places underlain by clay. In these lower reaches, *N. lighti* occurs rather scatteringly and does not reach large size, a possible result of the extremely great salinity fluctuations, or of predation by wading birds at times of low tide.

Location 2 is a pool some 25 yards from the normal brink of the river, confluent with it at times of high water, but usually isolated during the summer months. *Neanthes* occurs abundantly in firm sand with an admixture of clay, black with organic matter. The salinity of the pool is variable, but is usually high, especially in summer.

Location 3 is a muddy channel winding through the *Salicornia* marsh to the south of Location 2, from which it is separated by a low roadway. This channel is isolated from the estuary except at times of flood, or at occasional high spring tides when the river mouth is open to the sea. The salinity is usually over 50% that of sea water, and may rise much higher in summer, when the channel dries up to a series of tepid pools. The bottom is of soft sandy mud, with areas of clay, quite black and foul. *N. lighti* is abundant in such areas as do not dry up entirely in summer, commonly reaching a length of 60–65 mm. preserved (Hartman, 1938, reports 45 mm. as the maximum length). The worms occupy Y-shaped burrows extending 5–8 inches into the black mud, where presumably the salinity fluctuates far less than in the overlying water, but they are by no means insulated from this water, since the light-colored oxidized sand of the burrow walls indicates that respiratory irrigation is maintained in these passages.

The middle reaches of the estuary may be said to extend to two miles above the bridges (Fig. 1), at which point salinity variations due to tides are distinct but not extreme. At the bridges (1¾ miles from the mouth) observations made on a single day showed that salinities varied from 3.7 to 40.4% sea water over the tidal cycle. Because of the sparse population of *Neanthes* in this area, regular salinity records were not kept. Above the bridges, the estuary is narrowed and winding. Cat-tails, tulles, and willows replace the *Salicornia* and sedges of the lower reaches. By three miles from the mouth, the estuary has taken on a superficially almost fresh water aspect. Whereas the lower reaches in dry years contain *Enteromorpha, Ulva,* and encrusting green algae, the upper reaches, 4–5 miles from the mouth, contain masses of *Chara* and duckweed (*Lemna*). *Potamogeton* is abundant in both areas. Four locations in the upper reaches and the head of the estuary were studied, but at only the first of these (Location 4) were regular salinity records obtained.

Location 4 represents a widened portion of river bed with sandy bars on which *N. lighti* was extremely abundant following the dry winters of 1946–7 and 1947–8, when the area was undisturbed by flood waters and the salinity varied little. At this location, in 1948, as many as 40 adult worms could be picked from a single shovelful of sand. At times of high water, it might be difficult to obtain many individuals, while floods in the winters of 1949 and following years have swept out much of this formerly productive area. Salinities at Location 4 have remained below 3% of sea water except that as the river flow slackens in the spring occasional tidal influxes have been observed to raise the salinity temporarily to 20% of sea water or more. Conditions following the outflow of early 1952 have entirely altered this and the following upstream locations; these descriptions apply to the period of relative stability prior to 1952.

Location 5 lay about one-half mile upstream from Location 4, and represented the limit of *N. lighti,* where small numbers of reproducing worms were collected in July, 1949 (chlorinity 2.2% of sea water). This collecting spot was washed out by flood waters early in 1950.

Location 6 is about a half mile above Location 5. The intervening stretch of river was lined by cat-tails and was so narrow that in spots a skiff could be poled through only with difficulty. A current was generally perceptible in all but the driest weather, hence this region could have been considered the "head" of the estuary. Chlorinities up to 1.76% of sea water have been recorded from Location 6, which represented a sandy area just below the outfall of a drainage ditch. Although topographic maps (compiled in 1947) show the limit of permanent water in the vicinity of Location 5, I have regularly been able to take a skiff another mile upstream, there being a channel several feet deep, much narrowed by encroaching rushes and floating weeds. No *Neanthes* have ever been found at Location 6, although the substrate seems entirely suitable. However, this area is separated from Location 5 by a very muddy stretch which may have acted as a barrier to migration.

Location 7 lay at what was the limit of boat passage prior to 1952, almost a half mile from Location 6, a little over 1.5 miles above Location 4. A slight current was usually perceptible. Chlorinities up to 1.4% of sea water have been obtained in summer. No *Neanthes* have been found, and the aspect was that of a fresh-water environment. Above this point the river channel consisted of a series of pools, separated by impenetrable beds of cat-tails.

Flood waters in the winter of 1952 swept out the masses of vegetation above Location 7, widening and deepening the channel, and permitting a boat to be taken upstream for at least another mile even at low water. Locations 5 and 7 are no longer recognizable, some bars having been swept away, others covered with many feet of sand.

The above account of conditions prevailing in the summer months at seven Locations may be amplified by presenting salinity fluctuations at three of these: Location 3, representing an area of variable but high salinity; Location 4, an area of uniformly low salinity (oligohaline); and Location 1 in the lower estuary, the area subjected to the greatest salinity changes. The data on these three representative locations have been plotted in Figure 2 (bottom) where they may be compared with each other and with rainfall and runoff at corresponding times. It is apparent that a degree of regularity in the annual pattern of salinity variation existed at each location prior to the flood year 1951–52, and that in the latter water-year conditions were profoundly altered.

At the start of field observations in 1948 (Fig. 2, A) the lower reaches (Loc. 1) were almost as fresh as the upper reaches (Loc. 4), but the isolated channel in the *Salicornia* marsh (Loc. 3) to the south of the river had a salinity of 90% of sea water, and Location 2 (not plotted) was intermediate. Early fall rains (B) in 1948 temporarily dropped the salinity at Location 3 to 20% of sea water, a loss soon largely made up by seepage and mixing. The winter rains (C) of 1948–49 produced a general lowering of salinity, culminating in early March in an inundation of the *Salicornia* marsh by nearly fresh water, followed by a break-through of the river to the sea (D). This was followed by tidal influxes (E) which caused the lower reaches (Loc. 1) to alternate between fresh and salt water, while the pool near the river (Loc. 2) became filled with sea water. With the cessation of heavy rains the flow in the river dropped to a point which permitted the reëstablishment of the bar across the mouth early in May. Although occasional high tides spilled across this bar, the lower river continued to freshen until the winter of 1950 (F, G). The pool at Location 2 remained cut off from the river throughout the summer of 1949 and retained a moderate salinity despite its proximity to the brackish river until the fall rains diluted it and eventually put it into shallow communication with the river in November 1949. The more remote pools of Location 3 became steadily concentrated by evaporation throughout the summer (F), reaching a salinity of 110% that of sea water, and then were diluted by fall rains (G).

Special interest attaches to the salinity at Location 4 during the period in 1949 when the lower estuary was subjected to tidal exchange. With the opening of the bar, the water level at Location 4 dropped until many *Neanthes* burrows were completely out of water, the surface of the sand becoming dry and sun-baked. Although the worms burrowed deeply to some 18 inches, a gradual depopulation of such exposed bars occurred; whether by lateral burrowing to lower areas or by dying off could not be determined. With each tide, but lagging by several hours, the water level at Location 4 rose and fell about a foot. That this did not represent an influx of ocean water, but merely a backing-up and draining of fresh water, was established by repeated salinity determinations showing no difference between high and low water. In that year the bar closed in May, before the river flow became minimal. During the summer of 1949, Location 4 showed a slow rise in salinity to nearly 3% of sea water, with a gradual reduction after the fall rains (H). The winter rains of early 1950 resulted in the marshes along the lower river becoming flooded with nearly fresh water before the opening of the bar on February 8 (I). High tides coming during the succeeding period of lessened rainfall flooded the marshes with ocean water (J), raising the salinity at Location 3 to approximately 50% of sea water, but there was no indication of any salinity rise at Location 4. After closure of the bar at the beginning of March (1950), the river remained so high as to be confluent with the pool at Location 2, and both of these locations continued to freshen throughout the spring of 1950 (K). The hot late summer raised the salinity at Location 3 to 109.5% of sea water (L), but that at Location 2 remained low, without in any way seeming to harm the *Neanthes* present at either station, although reproduction seemed to be prevented by water temperatures near 30° C. on sunny days.

The fall of 1950 was marked by excessively heavy rains (M), which inundated Location 3 with nearly fresh water before the bar was opened on November 17. During December, high tides flooded the lower marshes with salt water (N), and as the river current slackened, eventually sent surges of sea water several miles upstream (O), where at Location 4 a salinity 21% that of sea water was recorded on January 6, 1951. Although by January 15 the chlorinity of the river water at this point had dropped to 2.4% of sea water, a value of 11.7% of sea water was obtained from water seeping into a hole in the sandbar from which worms were dug. The river mouth had practically closed by February 21, trapping within the lower estuary approximately 60% sea water, suggesting a considerable degree of mixing. By May, 1951, salinities at Location 4 had dropped to the usual 2.2% of sea water (P). A considerable salinity rise at Location 4, the result of spring tides at a period of slackening river flow, has been observed in two separate periods in four years, hence it very probably occurs in other years, and would seem to account for the higher "residual" chlorinities commonly encountered in water obtained from holes dug in the banks of the upper estuary. This may be an important factor in permitting *Neanthes* to survive beneath nearly fresh water.

The summer of 1951 was exceptionally cool and cloudy (Q). Salinity records were unavoidably interrupted, but the river remained high, confluent with the pool at Location 2, while the water in channels back from the river (Location 3) apparently did not become as concentrated by evaporation as in typical summers. Heavy December rains (R) flooded the lower marshes, and the river broke through the bar on December 30, 1951. A visit to Location 4 on January 1, 1952 showed a swirling muddy torrent pouring to the sea. Such sand bars as could be approached were found to be shifting masses of quicksand, indicating a heavy loss of *Neanthes* from this area. The great fresh water discharge during early 1952 signaled the end of the previous ecological distinctness of Locations 3 and 4. The flow into the lower reaches was high enough to inundate the *Salicornia* marshes with water of low salinity (S)—at least there is no evidence of the flooding of the marsh with sea water from a high tide at a time of slackening river flow as had been the case in previous springs, although the Location 2 pool did receive tidal influxes. The result was that Location 3 remained at a very low salinity, and did not in the entire summer of 1952 attain a salinity in excess of 27% sea water (S, T). Concommitantly *Neanthes* virtually disappeared from Location 3; only a few very small worms were found by careful search in July. However, Location 2, which had received influxes of sea water, retained the usual pattern of high spring and summer salinity (reaching 135% of sea water on July 30), and continued to support a normal population of *Neanthes.* It is difficult to avoid the impression that the worms of Location 3 suffered heavy mortality as a result of several months of salinities below 20% of sea water.

In the case of Location 4, the destruction of the worm population was nearly complete (only a single worm rewarded several hours of intensive search on July 31, 1952). But here the loss may be more attributable to the extensive erosion, widening, and deepening of the channel at Location 4 by several months of high stream flow than to adverse effects of salinity change. With the slackening of river flow in the spring of 1952 (T), and continuing through the summer (U), tidal effects became regular in the opened river channel. The mouth of the river remained open throughout the summer of 1952, apparently as a result of tidal scouring, for the flow was not much in excess of normal. Possibly wave action in the summer months is insufficient to throw up the barrier bar if a high discharge by the river in the spring has prevented formation of the bar at the usual time. As a result, the salinity at Location 4 rose and fell more or less regularly, a range of 15 to 30% sea water being noted in the course of a single moderate tide; probably the larger tides had considerably greater effects.

The fact that some worms evidently survived the summer of 1952 in both Locations 3 and 4, and survived in normal abundance at Location 2, suggests that in future years of low rainfall we may again see conditions stabilized and new populations of *Neanthes* developed as before. The evolutionary interest pertaining to such massive depopulations followed by repopulation from a few survivors is obvious, and a re-study of physiological adaptions in the new populations, if such arise, should be made to extend the preliminary studies carried out upon the past populations.

FAUNAL ASSOCIATES OF NEANTHES LIGHTI

In the summers of 1949 and 1950, before the stabilized "dry-year" pattern of the Salinas estuary had been upset, collections of the fauna at the several stations were made. Although conditions have in some instances been altered drastically, the following notes give a picture of the estuarine fauna in a condition as near equilibrium as is likely to be found. Since the range of *N. lighti* shows such marked variation in salinity, and includes areas with a distinctly fresh water aspect, a tabulation of the animals associated at a given location may be of value in estimating the relative extent of the penetration of this worm into "fresh" water. The statement of Hartman (1938) that *N. lighti* may occur in freshwater pools, as judged by the associated fauna of insects, etc., should be checked by a study of actual salinities. As is shown below, the faunal associates of *N. lighti* in the Salinas River include both fresh water and brackish water types.

Location 1: Most common were the isopod *Neosphaeroma oregonensis,* and the amphipods *Corophium spinicorne* and *Anisogammarus confervicolus.* The chief planktonic crustacean was *Neomysis mercedi,* while insects were represented by corixids and dipteran larvae. The openness of the area and the abundance of waders and sea-birds may contribute to a general paucity of fauna.

Locations 2 and 3: These contain in general the same fauna as Location 1 except the *Neomysis* is absent from both and *Corophium spinicorne* scarce at Location 3, especially at periods of high salinity. The small snail *Amnicola* occurs in large numbers on *Potamogeton* and other plants at Location 2.

Location 4: At this and the following points, collections were made from among cat-tails as well as from the adjoining sand, so that the habitats examined in the upper reaches are comparable. The fauna included the following typically fresh-water forms: *Physa, Chironomus, Plumatella, Hyalella azteca,* dragon-fly larvae, several dipteran larvae, bellostomid and corixid bugs, and beetle larvae. In addition, the following forms of marine affinities were well represented: *Corophium spinicorne, Anisogammarus confervicolus, Neosphaeroma oregonensis,* and *Neomysis mercedi.* Careful search revealed no planarians but a few leeches (*Placobdella fusca*).

Location 5: A small coarse sand beach contained small numbers of breeding *Neanthes* in July, 1949 (salinity 2.2% sea water). Collections in this area included essentially all the fresh water types encountered at Location 4, plus the snail *Gyraulus,* ephemerid and damsel-fly larvae and a few small planarians. *Corophium spinicorne* and *Neosphaeroma* were present but scarce. *Anisogammarus* was absent.

Location 6: Below the point where fresh water drainage ditch empties into the river, a sand bar suitable for *Neanthes* was examined, but found to harbor only chironomids and *Corophium stimpsoni,* the latter breeding and present in large numbers. Since the bottom appeared suitable and the chlorinity possibly adequate (1.75% of sea water), the absence of *Neanthes* may have been due to the long stretch of river above Location 5 which lacked suitable bottom for these worms. The fauna also differed from that at Location 5 in the absence of *Neosphaeroma* and of *Corophium spinicorne.* The replacement of the latter by *C. stimpsoni* is of especial interest. Shoemaker (1949) states that *C. spinicorne* is the only *Corophium* recorded from the fresh waters of America, but the presence of *C. stimpsoni* in the freshest part of the Salinas estuary suggests that the later species is actually more tolerant of fresh water than is *C. spinicorne.*

Location 7 was at the limit of boat passage, approximately ½ mile from Location 6 (2 miles from Location 4). The fresh water fauna previously encountered were here in abundance, including numerous leeches and very abundant large planarians (*Dugesia* sp.). The only representative of the "marine" fauna was *Neomysis,* present in great numbers. Chlorinity was 1.4% that of sea water. The bottom was muddy and no *Corophium* were taken.

This cursory account suffices to show that the range of *Neanthes lighti* coincides in a general way with those of *Neosphaeroma oregonensis, Anisogammarus confervicolus,* and *Corophium spinicorne,* all of which are characteristic brackish water species. Conversely, it appears that *N. lighti* occupies a region which overlaps only slightly the ranges of such fresh water forms as planarians or leeches, although numerous fresh water insects, as well as the snail *Physa,* extend into the less saline portions of the range of *Neanthes.* It would appear on the basis of these scanty collections that *Corophium stimpsoni* may be restricted to waters less saline than those tolerated by *Neanthes lighti,* but final judgment on this point should await a detailed study of the distribution of the several species of *Corophium* within brackish waters. Certain forms, such as *Neomysis mercedi* and the groups of the corixids and chironomids, have a much wider salinity tolerance than *N. lighti,* and are not so useful as indicators in this situation.

Discussion

While Figure 2 upon first inspection seems to reveal a situation little short of chaotic, the observations over the past several years permit certain generalizations concerning the conditions of life in a small California estuary. There are, most strikingly, wide and irregular salinity variations in the lower reaches, these being most marked in the lower river channel itself, where long periods of quiet water of low salinity are interspersed with weeks or months of tidal action which may result in a daily salinity shift from 3% to 100% of sea water, as well as exposure to air and to predators. It is perhaps significant that in this area of most extreme change and exposure hazard, *Neanthes lighti* is least abundant.

These worms have been more consistently abundant, larger, and more vigorous in two general situations, differing in opposite senses from the lower estuary proper. The following remarks apply to conditions prior to the upset of 1951–52, which is considered to have been a decidedly abnormal season. At Location 3 there has usually been a salinity range of 30–40% to above 100% that of sea water. Owing to the tendency of fresh water to layer out above salt water, and for substrata of high salinity to be little affected by submergence beneath fresh water for relatively short periods (Reid, 1930, 1932), it does not seem likely that worms in their burrows in the muddy channel at Location 3 are ever abruptly exposed to salinities as low as are measured in the river itself at times of flood, or at most for very limited periods. Worms from this area can be adapted to chlorinities equivalent to 1% of sea water; hence salinity variations at Location 3 are not extremely severe. Temperatures of 30° C. in summer seem not to injure the adult worms, but result in failure of fertilization and/or development of embryos (Smith, 1950).

In marked contrast has been the prevailing low salinity at Location 4 in normal years. Over most of the period of observation, chlorinities have varied from about 3% of sea water down to as low as 0.25% at times of flood. Worms in their burrows perhaps receive protection against the lower salinities of the flowing water above them as a result of "residual" salinity in the sand of the banks and bottom. It has commonly been noted that worms collected at times of flood become much swollen if retained in river water, but survive better if kept in the sand from which they were dug. Such worms tend to form distinct "jackets" of mucus and sand, which may offer some protection. It has also been found best to keep worms collected from brackish water at room temperature, since refrigeration apparently retards active osmoregulation to such a degree that swelling and death may occur. Mixing from the lower reaches and occasional tidal influxes of sea water probably serve to maintain the slight salinity required for survival in such nearly fresh-water areas in normal years.

It is apparent that *N. lighti* is a brackish-water type which has not completed the transition to a strictly fresh water habitat. From a study of conditions in the Salinas River it is not possible to say whether this transition could be made. From the fact that the range of *N. lighti* seems to coincide with that of such forms as *Corophium spinicorne* and *Neosphaeroma oregonensis,* but does not extend so far upstream as that of *Corophium stimpsoni* or *Neomysis mercedi,* we may judge that it is actually limited in its osmoregulatory abilities. Furthermore, the dropping out of such typically fresh water forms as odonata larvae, planarians, and leeches as the fresh water fauna approaches the upper limit of *N. lighti* suggests that the salinities required by *N. lighti* are not inappreciable, inasmuch as they may limit the seaward extension of these fresh water animals.

Physical barriers to the upstream spread of *N. lighti* appear serious, but are difficult to evaluate precisely. Recurrent rushes of flood water have been observed to sweep away well-populated sand bars, or to cover productive areas with thick layers of barren sand. Thus the bar at Location 5, which in 1949 marked the upstream limit of *N. lighti,* was completely washed away in February, 1950, and the area has not been repopulated. Another factor which may have limited the spread upstream beyond Location 5 is the fact that the stream above this point became thickly bordered by cat-tails growing in very soft black mud. *N. lighti* seems to require a sandy substrate for its burrows, and has never been taken in the sort of mud found for the half mile above Location 5. There is usually a perceptible current in this area, even in summer in dry years, which may have further discouraged upstream migration of young worms.

The nereid population of the upper reaches has been in several senses isolated from that of the *Salicornia* marshes: First, by the fact that the normal range of salinities at Location 4 does not (except for infrequent and brief periods) overlap the range of salinities at Location 3. Second, by the presence of intervening stretches of river physically poorly suited for large populations of *Neanthes.* Third, by the fact that *N. lighti* appears to consist of self-fertilizing hermaphroditic viviparous individuals, which do not produce widely-dispersing planktonic larvae. The problem of whether or not these populations constitute actual physiological races requires more detailed study. Preliminary studies have been made to ascertain this point, but since the studies cannot be confirmed until conditions in the estuary have become stabilized and new populations developed, only an abstract of these studies need be included here. In this work, the respiration of worms from Location 4 was compared with that of worms from Location 3. The method used was to adapt worms from each area gradually to a series of different salinities (1, 5, 10, 50, 100, and over 100% of sea water), and to maintain them (with feeding) at these salinities for three or more weeks to ensure full adaptation. It was considered that the worms became nearly as fully adapted to the test salinities as their physiological constitutions would permit. It was found that worms from Location 3, adapted to salinities 5% or less of sea water showed a higher respiration on the average than did worms from Location 4 adapted to identical media. Since a number of variables, including size, had to be taken into account, the results, although apparently significant, are considered indicative of, rather than conclusive evidence for, the existence of physiologically distinct races of *N. lighti* in different parts of the Salinas estuary. The drastic reduction of the populations in question following the hydrographic upset of 1951–52 further suggests that these populations were physiologically specialized or distinct. Because the upset of stable conditions will delay completion of these studies for some time, until the estuary stabilizes, the work will not be reported in detail until it can be confirmed.

SUMMARY

1. Salinity and salinity changes have been studied over the range of the polychaete *Neanthes lighti* Hartman in the estuary of the Salinas River, California, during the years 1948–1952.

2. Not only are mean salinities different in various parts of the range of *N. lighti,* but each locality has a characteristic annual pattern of salinity variation depending upon fresh water discharge into the estuary.

3. The pattern of salinity variation is traced through years of low and moderate rainfall, and its upset during the flood season of 1951–52 is reported.

4. Worms inhabiting marshy areas near the river mouth are exposed to variable but high salinities (40 to over 100% of sea water); those in the upper reaches endure uniformly oligohaline conditions (2–3% of sea water) for most of the year. Either of these areas is more favorable than the lower reaches of the estuary that receive full tidal exchanges for part of each year.

5. Over its range, *N. lighti* is associated with such typically brackish water species as *Neosphaeroma oregonensis* (Dana), *Anisogammarus confervicolus* (Stimpson), and *Corophium spinicorne* Stimpson. Its range only slightly overlaps the ranges of such fresh water forms as *Dugesia* sp., leeches, or odonata larvae. *Corophium stimpsoni* Shoemaker is found in water fresher than that inhabited by *N. lighti* or the crustaceans associated with it.

6. The populations of *N. lighti* of the upper reaches are ecologically and reproductively isolated from those of the more saline marsh channels, and some evidence for the existence of physiological distinctness between these populations has been found.

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QUANTITATIVE STUDY ON THE REGIONAL DISTRIBUTION OF PENTOSE NUCLEIC ACID IN THE GASTRULA AND NEURULA OF TRITURUS

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The aim of the present study is to estimate chemically the amount of pentose nucleic acid in the different prospective regions of the ectoderm and mesoderm in the early gastrula and the early neurula of Amphibia, and to obtain quantitative information about its spatial distribution and its change during gastrulation. The eggs of *Triturus pyrrhogaster,* which were found to satisfy special demands of our study, were used exclusively. In this material the prospective significance and the morphogenetic movement of the different regions have been worked out in detail (Nakamura, 1942) and operative separation of these regions can be successfully carried out with techniques developed in this laboratory.

MATERIAL AND METHOD

The estimation was carried out on the following developmental stages: (1) gastrula with beginning blastopore, corresponding approximately to stage 11 of Okada and Ichikawa's table of *Triturus pyrrhogaster* (1947) and to stage 10 of Harrison's table of *Amblystoma punctatum;* (2) neurula, shortly after the formation of the neural folds, corresponding to stage 16 of Okada and Ichikawa's table and to stage 14 of Harrison's table.

Following the removal of the capsule and vitelline membrane the embryos were operated on in Holtfreter's solution on a Schotté ring using the glass needle and glass spherule. For a better separation of the germ layers the operation dish was kept cold with ice. During the operation utmost care was taken to obtain isolates of pure prospective significance, free from contamination by cells from the adjoining regions and underlying layer.

In the gastrula the following regions were isolated for estimation:

(a) The prospective ectoderm (A, Fig. 1a), consisting of the prospective neural plate (the cross-hatched part of A) and the prospective epidermis (the plain part of A). In a special series the prospective neural plate and the prospective epidermis were estimated separately.

(b) The dorsal marginal zone (B, Fig. 1a), containing the prospective notochord and the dorsal half of the prospective somites. The prospective pre-

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chordal material, which is continuous with the yolk-rich endoderm of the pharyngeal wall, without a clear boundary, was not included.

(c) The lateral marginal zone (C, Fig. 1a), containing the ventral part of the prospective somites and the prospective lateral plate.

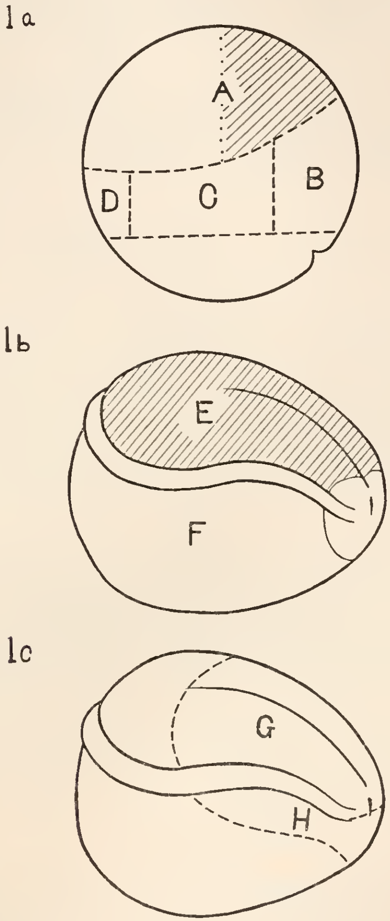

FIGURE 1. Schemes for isolation of samples for PNA estimation. 1a: Gastrula; A: prospective ectoderm (the cross-hatched half is the prospective neural plate, the plain half the prospective epidermis); B: dorsal marginal zone; C: lateral marginal zone; D: ventral marginal zone. 1b: Neurula; E: neural plate (cross-hatched area); F: epidermis (neural fold, and area surrounding the blastopore are omitted). 1c: Neurula; G: dorsal mesoderm; H: lateral and ventral mesoderm.

(d) The ventral marginal zone (D, Fig. 1a), containing the prospective blood island and adjoining ventral mesoderm.

From the neurula the following regions were estimated:

(a) The neural plate (E, Fig. 1b). The neural folds were not included. Also the most caudal one-fifth of the neural plate which contained the prospective somites (Nakamura, 1942) was omitted. The cells of the archenteric roof, which were sticking to the internal surface of the neural plate, were removed very carefully with the finest glass spherules.

(b) The epidermis (F, Fig. 1b). Only very careful operation gave pure epidermis without attached mesoderm cells, which were more difficult to remove than the archenteric roof cells. Of course only pure isolates were used for the estimation.

(c) The archenteric roof (G, Fig. 1c). Most of the prechordal plate was omitted. No endodermal pharyngeal wall was included. This region corresponded to region B and the dorsal part of region C of the gastrula.

(d) The latero-ventral mesoderm (H, Fig. 1d); the mesoderm underlying the epidermis. This is derived from region D and the ventral part of region C of the gastrula.

For the chemical estimation of PNA, Schneider's technique (1945) was adopted. The intensity of orcinol reaction was read by a Pulfrich spectrophotometer. Interference by DNA contained in the "PNA-fraction" was found to be negligible. PNA concentration was expressed in micrograms of phosphorus per milligram of the total nitrogen. Total nitrogen was estimated by the method of Levy and Palmer (1940) on the residue after the nucleic acid extraction. In the principal series six separate determinations were carried out for each region of both stages, while in the additional series four separate determinations were made. Thirty-five to 45 pieces of isolates made up the sample for one estimation.

TABLE I

PNA Pγ per mg. N of the different prospective regions of the early gastrula and the early neurula

$\bar{X}$: mean; $S_{\bar{x}}$: standard deviation of the mean; **: F_0-value significant at 1% level; *: F_0-value significant at 5% level but not so at 1% level; °: the same value not significant on 5% level.

Stage	Prospective regions	PNA Pγ/mg. N $\bar{X} \pm S_{\bar{x}}$	Ratio of variances F_0
Gastrula	(A) Prosp. ectoderm	9.6±0.60	28.93**
	(B) Dorsal marginal zone	6.0±0.30	11.42*
	(C) Lateral marginal zone	4.8±0.11	0.25°
	(D) Ventral marginal zone	4.6±0.33	
	(A′) Prosp. neural plate	11.5±0.28	10.98*
	(A″) Prosp. epidermis	9.7±0.47	
Neurula	(E) Neural plate	13.8±0.68	7.88*
	(F) Epidermis	11.1±0.66	11.83**
	(G) Dorsal mesoderm	7.7±0.73	0.01°
	(H) Latero-ventral mesoderm	7.6±0.74	

TABLE II

Comparison of PNA Pγ/mg. N of prospective regions of the gastrula and neurula. Compare with Table I

Prospective regions	Ratio of variances F_0
Prospective neural plate of gastrula Neural plate of neurula	6.9*
Prospective epidermis of gastrula Epidermis of neurula	2.67°
Prospective ectoderm of gastrula Neural plate of neurula	20.67**
Prospective ectoderm of gastrula Epidermis of neurula	2.83°

RESULTS

The principal data obtained are summarized in Table I.

Gastrula. The prospective ectoderm (A) showed the highest value, while the dorsal marginal zone (B) gave a value significantly less than the former. The dorsal marginal zone, the organizer region, in its turn showed PNA content higher than the lateral and ventral sections of the marginal zone. This difference was significant at the 5% level, but hardly so at the 1% level. The latter two sections failed to give any significant difference.

In the additional series PNA content of the prospective neural plate and the prospective epidermis was estimated (Table II). The results showed a difference significant at the 5% level in favor of the prospective neural plate.

Neurula. The neural plate (E) showed the highest value, significantly higher than the dorsal and latero-ventral mesoderm of the same stage (G and H). The difference between the neural plate (E) and the epidermis (F) was found to be significant at the 5% level, but not at the 1% level. No significant difference could be detected between the dorsal and latero-ventral mesoderm.

DISCUSSION

The present data show clearly that in both stages examined the ectoderm contains more PNA than the mesoderm, if the amount is calculated on the over-all nitrogen base. The organizer region, *i.e.*, the dorsal marginal zone of the gastrula and the archenteric roof of the neurula, contain significantly less PNA than is contained in the ectoderm in both stages. Within the ectoderm the neural plate and its prospective region seem to possess more PNA than the epidermis and its prospective region, although the difference obtained is significant only at the 5% level. Thus, our data make it very probable that the apex of the gradient of the substance throughout both germ layers is present in the material of the neural system in the stages studied. Comparing the data on ectodermal regions of both stages it might be pointed out that during gastrulation an accumulation significant at the 5% level can be detected for the neural plate region, but not for the epidermis region.

As to the marginal zone our data show a difference significant at the 5% level between its dorsal section and the rest of the zone in the gastrula. However, in the neurula no significant difference can be shown between the dorsal and latero-ventral mesoderm.

If we compare the data obtained from different regions with the inductive effect of these regions on the presumptive ectoderm, it is clear that the simple over-all PNA concentration of any germ region as such does not decide the inductive capacity of that region. The epidermis of the neurula is non-inductive and the ectoderm of the gastrula is very weakly if at all inductive, but they contain more PNA than the organizer itself. In the early neurula the archenteric roof has stronger inductive effect but not the latero-ventral mesoderm. However, they all have about the same amount of PNA. Furthermore this amount is decisively less than that of the non-inductive epidermis.

As to the spatial distribution of PNA within the different prospective regions of the amphibian embryo, until now only data based on the observation of the histochemical section have been available. The general features of the gradient as demonstrated by histochemical techniques by Brachet (1942, 1947), such as the animal-vegetal and dorso-ventral gradient, are mostly in accord with our present data. However, no indication was obtained in the present study of a high concentration of PNA in the organizer. The present author studied the PNAase-sensitive basophilia in embryos of *Rhacophorus schlegelii* (anura, pigmentless eggs; Takata, 1950), *Triturus pyrrhogaster* and *Megalobatrachus japonicus* (urodele, pigmentless eggs; Takata, unpublished results). During gastrulation the apex of the gradient was found always in the prospective neural plate. The dorsal marginal zone was stronger in intensity than the lateral and ventral marginal zone, but decisively weaker than the ectodermal regions. These observations are in good accord with the present data and there seems to be no ground to support the idea that the organizer represents the apex of the PNA gradient, if its value is calculated on the basis of over-all nitrogen. However, it is still an undecided question whether the PNA concentration of the active protoplasm (*i.e.,* PNA calculated on the basis of the non-yolk nitrogen) has its highest value in the organizer region.

Insofar as data on total nitrogen basis are concerned we did not find any indication for decrease of PNA in the invaginating archenteric roof, as suggested by Brachet from his histochemical sections.

Comparing the data on two stages, a general increase of PNA content in both layers during gastrulation is evident. This rise seems to be in accord with the data on the total PNA amount of amphibian embryos during the early development, where more or less clear increase of the value beginning from gastrulation was noticed (Brachet, 1941; Steinert, 1951).

SUMMARY

1. PNA content of prospective regions of the ectoderm and mesoderm of the early gastrula and neurula of *Triturus pyrrhogaster* was estimated by the technique of Schneider (1945) and expressed in microgram phosphorus per mg. nitrogen.

2. At both developmental stages PNA content was higher in the ectodermal regions than in the mesodermal regions. During gastrulation, an increase in PNA amount was noticed for most of the regions studied.

3. In the gastrula the highest amount of PNA was found in the ectoderm (prospective neural plate) but not in the organizer region. In the marginal zone the dorsal region showed an amount higher than that of the lateral and ventral marginal zones.

4. In the neurula the neural plate showed the highest value. The main part of the archenteric roof contained less PNA than the epidermis. No significant difference could be detected between the values of the archenteric roof and the rest of the mesoderm.

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ABSTRACTS OF PAPERS PRESENTED AT THE MARINE BIOLOGICAL LABORATORY

1953

ABSTRACTS OF SEMINAR PAPERS

JUNE 30, 1953

The structure of ribonuclease.[1] DOROTHY WRINCH.

Preliminary X-ray studies of crystalline proteins have been made by many investigators from which only broad inferences regarding symmetry and volume of the protein entities can be made. However, for a few crystalline proteins, intensity data have been obtained. In such cases information regarding the structures of the proteins can be obtained if, and only if, it proves possible to interpret the intensity map or its equivalent, the vector map. The fundamental question relating to a particular protein entity for which it is feasible to look for answers in intensity or vector maps at this time concerns *the type of atomic pattern* adopted by the sets of interlocked N-C_α-C monomers which constitute the skeletons of the molecules of the entity in question.

A more detailed study of the vector maps of ribonuclease given by Carlisle and Scouloudi (1951, 1953) now confirms the suggestion (Wrinch, 1952) that this protein does not have the classical type of structure comprising a bundle of polypeptide chains and that, on the contrary, the data lend themselves to interpretation in terms of skeletons which are genuinely three-dimensional in character. The nature of the vector maps suggests that these structures are indeed globulite, *i.e.,* more or less equi-dimensional in shape, that they are cage-like and that they have low-density interiors. From Perutz's X-ray data on horse hemoglobin (1949), corresponding conclusions were reached (Wrinch, 1952, 1953). Comparing the results we encounter the striking fact that, for these two different proteins, the X-ray data may be interpreted in terms of skeletons which are not only of the same type but are even of about the same dimensions, having diameters of the order of, say, 10–15 Å.

An Approach to the problem of DNA constancy.[2] W. S. VINCENT.

The DNA (desoxyribonucleic acid) content of individual nuclei is usually determined by microphotometry of the Feulgen stain. Such measurements usually give, within one ploidy class, a normal distribution curve with a variation of about 30% of the mean. If one measures the dye bound in each of the two nuclei of a binucleate cell one might expect to sensibly control variations due to spatial effects of fixation and staining. The variation between two nuclei within a single cytoplasm should be less than that found between two randomly located nuclei if errors due to staining and fixation procedures are present. Variation due to instrument errors can be determined by repeated measurements on the same nucleus.

Tissue from the median lobe of a mouse liver fixed in alcohol-acetic, formalin-sucrose and Zenker-formol was hydrolyzed and stained for maximum color development in a standardized Feulgen procedure. Two measurements were made on each nucleus of fifteen binucleate cells from each fixative. Analysis of variance and calculation of the distribution of the variation gave the following results: Variation (1) due to measurement, 2%; (2) due to fixation, 3%; (3) between nuclei in the same cell, 47%; (4) between nuclei in different cells, 48%.

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[1] This work is supported by the ONR under contract with Smith College.

[2] Supported by AEC project 1187, administered by Dr. John W. Gowen.

Two possible explanations for the finding that nuclei in the same cell have the same variation as nuclei in different cells are given: (1) The normal distribution curve of DNA content found in microphotometric measurements on a cell population is a true picture of the content of this substance in the individual nuclei. This explanation implies that the hypothesis of DNA constancy is true only in a statistical sense for a given population of nuclei. (2) An alternate explanation is that the Feulgen procedure is subject to random and as yet undefined variations in combining with DNA. Such an explanation negates the value of the Feulgen reagent in accurately determining DNA content of single cells.

Chemical and electron microscopic observations on the isolated mitotic apparatus. DANIEL MAZIA.

The method of isolating the mitotic apparatus from dividing sea urchin eggs that was described earlier by Mazia and Dan involved three steps: (1) preservation of the intact apparatus in subzero 30 per cent ethanol, (2) stabilization by means of an oxidizing agent, and (3) dispersal of the cytoplasm surrounding the mitotic apparatus by means of the detergent Duponol. Step (2) undoubtedly causes drastic alteration of the proteins and Duponol also is known to denature many proteins. The method has now been modified by the elimination of step (2), which is possible if a much gentler detergent than Duponol—digitonin—is employed to disperse the cytoplasm.

The newer and gentler method of isolation, applied to the eggs of *Strongylocentrotus purpuratus,* yields intact mitotic apparatus with the chromosomes as well as the spindles, asters, and centers preserved in a seemingly normal condition. The chromosomes retain their DNA, and the protein isolated from the apparatus also contains about 2% RNA, which had not been found in the earlier preparations. The mitotic apparatus isolated by the digitonin method is soluble in 1% sodium thiogylcollate at pH 11.5. At pH 11.0 the thioglycollate dissolves the spindle and the radiating fibers of the aster, leaving behind the centrosphere and that part of the aster immediately surrounding the centrosphere. It is thus possible to isolate this part of the mitotic apparatus.

Electron microscopic observation reveals the mitotic apparatus to be a gel in which the fibers represent regions of higher orientation.

JULY 7, 1953

Neuronal derivatives of the neural crest in the chick. WARNER S. HAMMOND.

Experiments in which the neural tube or the neural crest of the chick embryo were removed indicate that the appearance of sympathetic ganglia of the neck and trunk is dependent upon the presence of cells from the neural crest of the region. In addition, hindbrain crest is found to be the source of cells for the intrinsic ganglia of the heart, lungs and digestive tract.

Independent differentiation of preganglionic and postganglionic neurons of the sympathetic system is also indicated by the experiments.

Neurons of the dorsal root ganglia are absent following extirpation of the neural crest and/or neural tube of chick embryos up to 28 somites in age. Failure of these neurons to differentiate after neural tube removal may be due to loss of their connection to the central nervous system.

The migration of morphologically undifferentiated cells from the neural crest occurs in a longitudinal direction as well as a dorso-ventral one. The factors responsible for migration, localization and subsequent differentiation of crest cells into neurones remain obscure. Kappers' theory of neurobiotaxis does not appear to be applicable in this situation.

The regression-replacement cycle of hydranths in Obelia and Campanularia. SEARS CROWELL.

Continuous observations of rapidly growing, healthy colonies of *Campanularia flexuosa* show that individual hydranths are short-lived: after a few days a hydranth loses its form; its substance passes into the coenosarc of the colony; the hydrotheca drops off; and in a day or two a new hydranth, with a short pedicel, develops at the tip of the old pedicel. On the average, feeding hydranths persist for about 4 days at 21–22° C. and for about 7 days at 17° C. The process is orderly in that older hydranths undergo regression before younger ones. A five-day starvation period accelerated the regression cycle but did not alter the orderliness of the sequence.

Original data sheets of F. S. Hammett show that older hydranths undergo regression much more frequently than younger ones when colonies are warmed for 24 hours. Ordinarily commercial slides of Obelia not only show stages of regression and replacement but also indicate the orderliness of regression. Older parts of the stem have pedicels whose perisarcal annulations show several distinct thicknesses. These are the record left by several cycles of regression and replacement.

Brief observation of a few other hydroids suggests that a natural cycle of regression and replacement is common in calyptoblasts. Although hydranths of gymnoblasts undergo regression this is not known to occur under favorable conditions. This may be related to the fact that once a calyptoblastic hydranth is developed it does not grow while a gymnoblastic hydranth grows extensively after becoming a small feeding hydranth.

The naturally occurring cycle of regression and replacement may have some advantage in that attaching organisms are removed by shedding of the hydrotheca. Probably the principal significance lies in the fact that a colony is able to avoid senescence by replacing old with new, and is able, by resorption of the substance of the old, to do this without loss to the colony as a whole.

JULY 14, 1953

Potassium and sodium movements in rabbit polymorphonuclear leukocytes. H. G. HEMPLING.

Studies were carried out on the electrolyte metabolism of rabbit polymorphonuclear leukocytes obtained by intraperitoneal injection. Potassium content was found to be 105.1 ± 6.1 meq/kg. cell water, sodium 67.5 ± 11.1 meq/kg. cell water, and the water content 79.0 ± 1.14 per cent. Simple cytolysis of the cells in distilled water removed all analyzable K and Na, and ashing was found to be unnecessary. Cells in exudate fluid with an average K concentration of 2.0 meq/liter were found to lose K gradually over a 24-hour period at 37° C., replacing the K with a reciprocal amount of Na. Usually 50% of the cell K was lost during this time period. The addition of glucose was found to slow the rate of K loss, regardless if the glucose were added initially or at intermittent intervals. Studies on glucose utilization and hydrogen ion production were carried out in conjunction with electrolyte analyses. Protection by glucose against K loss could not be attributed to simple hydrogen ion exchange for extracellular K. Maintaining cell suspensions at 2° C. produced an enhanced loss of K as compared to losses at 37° C., described by the equation of a first order reaction. Per cent of cell K lost per hour was 5.76. An exact reciprocal of Na was gained. Systems allowed to lose K at 2° C. when placed at 36° C. took up K and extruded Na in a reciprocal fashion against concentration gradients. Approximately 100 meqs K/kg. cell water were transported in two hours from an environment of 3 meqs/liter.

Clotting of fibrinogen as a problem of protein chemistry. L. LORAND.

Studies on fibrinogen have established the basic principles involved in the clotting phenomenon. The alteration that fibrinogen undergoes prior to aggregation under the influence of thrombin has been characterized by a species specific change in the N-terminal residues of the protein (from glutamic acid to glycine in bovine, and alanine to glycine in human material) and the liberation of non-protein nitrogen (ca. 3%) in the form of fibrino-peptide. Fibrinopeptide is an acidic unit of the fibrinogen molecule and represents, at physiological pH, highly charged negative centers. The removal of these centers by thrombin would eliminate some repelling charges from fibrinogen, and make isoelectric sites on the protein that could serve as contact points for neighboring molecules. Once the fibrin particles are aligned in an oriented fashion, secondary forces would strengthen their association.

It is believed that the clotting of fibrinogen reveals a story that has a significance beyond the borders of blood coagulation itself, and it may be an indication of how certain principles are being utilized in building biological fibers. The mechanism represented by the fibrinogen-fibrin transformation may be a common pattern in the biogenesis of a number of proteins. The enzymically altered protein displays quite different properties from those of the primary one, although it differs very little from it in molecular and chemical constitution. In the case of fibrinogen the altered protein acquires the property of spontaneous fiber formation through loss of localized charges. The concept of such relatively minor change during clotting is in agreement with all experimental data so far.

The author wishes to acknowledge the cooperation of Dr. W. R. Middlebrook, with whom much of the above work was done.

Thermal inactivation of a bacterium-bacteriophage system. MAX A. LAUFFER, ANNE BUZZELL AND DAVID TRKULA.

The inactivation at 47° of the complex, *E. coli* B and bacteriophage T2r, was studied. The investigation was undertaken to see how the thermal stability of the complex varies as the growth of the bacteriophage progresses.

Bacteriophage particles were added to very concentrated suspensions of bacteria held at 5–10° in sufficient amount to give 10^{-2} to 10^{-3} phage particles per bacterium. Thirty minutes later the suspension was diluted with medium and incubated at 37°. After various times, samples were taken and immediately heated at 47°. Plaque counts were made after various times of heating. Inactivations at 47° followed first order kinetics. The rate of destruction of complexes incubated 0 minutes at 37° was 2.7×10^{-4} reciprocal seconds, a value very much higher than the rate of destruction at 47° of either free bacteriophage or free bacteria. The rate increased approximately two-fold for each three minutes of incubation at 37° for the first 12–14 minutes. At 12 minutes, bacteriophage stable at 47° began to appear, and by 16 minutes the phage count following heating at 47° exceeded the original amount. These results are interpreted to indicate premature lysis of complexes at 47°.

JULY 28, 1953

Glycolysis and potassium-sodium exchange in rabbit red cells exposed to ethyl and n-propyl carbamate. J. W. GREEN AND F. X. WAZETER.

Washed rabbit red cells, handled under sterile conditions, were incubated in 1% NaCl phosphate-buffered solutions at pH 7.00 and 34° C. for 48 hours in concentrations of 4 ml. of packed cells per 100 ml. of NaCl solution. To the suspensions were added 100 mg% glucose and varying amounts of ethyl or propyl carbamate. Suspension samples were removed at 12-hour intervals and determinations of Na and K, hemoglobin, glucose and pH were made. It was found that 0.3 *M* ethyl and 0.1 *M* propyl carbamate exerted a marked effect on accelerating Na-K exchange; cell K loss in the former suspension attained diffusion equilibrium within 48 hours, while in the latter somewhat more slowly. With both carbamates at these concentrations, the utilization of glucose was reduced to approximately 50% of that found in the control suspensions. Lower concentrations of both carbamates had either no effect or a slight stimulating effect on glucose utilization, while 0.5 *M* ethyl and 0.3 *M* propyl carbamate proved hemolytic after 10 hours of incubation. Potassium cell losses exceeded Na cell gains with 0.3 *M* ethyl and 0.1 *M* propyl carbamate. In the presence of ethyl carbamate the total cation content of the cells was reduced by approximately 25% and with propyl carbamate by half this amount at 48 hours. Cell volume changes did not reflect these cation shifts. The action of the carbamates on these cells is regarded as non-specific and suggests gelation of the cell contents.

A study of the oxidative activity of rabbit reticulocytes. F. R. HUNTER AND ALICE S. BAKER. No abstract submitted.

The influence of metabolic factors on the distribution of sodium and potassium ions in the green alga Ulva lactuca. GEORGE T. SCOTT AND HUGH R. HAYWARD.

The cells of the green alga *Ulva lactuca,* like those of most organisms living in a high sodium-low potassium medium, accumulate potassium and maintain within a relatively low sodium level. Experimental studies relating to the following were carried out on this form: 1. The influence of light and dark on the potassium and sodium distribution. 2. The influence of iodoacetate. 3. The influence of light and temperature on the exchange rate of potassium ion.

The alga when maintained in the dark for long periods loses some potassium and gains sodium, both effects being reversed on illumination. The presence of 0.001 *M* iodoacetate in the dark causes a marked progressive loss of potassium and gain of sodium. This influence of the inhibitor is completely prevented by light. Evidence for the penetration of the inhibitor in the light is presented, in that after the material is transferred from sea water containing the inhibitor in the light to running sea water in the dark a significant loss of potassium and gain of sodium occurs. The iodoacetate effects once established can be "washed out" by transferring the alga to light and running sea water without the inhibitor. Potassium reaccumulation begins after 24 hours whereas sodium ion is secreted out of the cell to the level of the control within four hours.

The exchange of cellular for environmental potassium was measured using K^{42} under conditions of illumination and darkness and at 20° and 30° C. The data indicate a complete exchange of cellular for environmental potassium which occurs at an increased rate under conditions of illumination and increased temperature.

In the interpretation of these findings it is emphasized that energetic coupling to metabolic processes must occur in the continual movement of ions against their concentration gradients. This energy presumably is derived from the degradation of phosphoglyceric acid, an intermediate formed in the breakdown of carbohydrate reverses, except in the presence of iodoacetate, or formed by photosynthesis even though the inhibitor be present.

AUGUST 4, 1953

On the mechanism of staircase and contracture in ventricular muscle. STEPHEN HAJDU. No abstract submitted.

Conduction in non-striated muscles. C. LADD PROSSER. No abstract submitted.

Structural and in vitro diffusion characteristics of intact and "desheathed" sciatic nerves from the toad (Bufo marinus) and from the bullfrog (Rana catesbiana). ABRAHAM M. SHANES.

Recently completed studies provide conclusive proof that the peripheral connective tissue sheath (epineurium or external perineurium) of the bullfrog sciatic completely determines the kinetics of diffusion and ion exchange in the intact nerve. Thus, the slow emergence of Na^{22} into inactive solution from nerves previously equilibrated in radioactive Ringer's is practically identical with that from sheaths containing only radioactive solution in their lumen. Consequently, the sheath must be removed when the permeability characteristics of the nerve fibers are to be examined. Unfortunately, desheathed bullfrog sciatics undergo a large (40%) increase in weight over a two-hour period in Ringer's, which renders them unsuitable for quantitative work.

The desheathed toad sciatic does not change significantly in weight. Moreover, the spike amplitude and the potassium content remain constant for at least 20–24 hours. Histological sections prepared by Dr. James B. Longley demonstrate that the bullfrog sheath is a mixture of at least two structures staining differently in Masson's. Both are completely removed by the desheathing operation. In the toad nerve, however, the sheath is composed of two relatively distinct structures: an outer one, which is relatively thick and takes up aniline blue, and an inner one, which is thin and stains with acid fuchsin; only the former is removed by the desheathing operation. Nevertheless, the apparent exchange (self diffusion) coefficient for the extracellular space is increased fifty-fold from the original value of about 7×10^{-7} cm.^{2} min.$^{-1}$ Although this is still 20 times smaller than in free solution, it is sufficient to permit study of the still slower ionic exchange across the membranes of the nerve fibers.

AUGUST 11, 1953

Ribonuclease activity in rat liver fractions. JAY S. ROTH.

Rat liver homogenate was separated into nuclei, mitochondria and supernatant using a modification of the technique of Hogeboom and Schneider. Cytochrome oxidase assays of nuclei and homogenates gave ratios of homogenate to nuclei activity of from 10 to 21, indicating that the nuclei were practically free of mitochondrial contamination.

Ribonuclease activity was determined in the three fractions and the original homogenate at 12 pH values from 4.47 to 8.52 by measurement of acid soluble P^{32} split from P^{32}-labeled yeast RNA. It was demonstrated that under the conditions of assay used there was no increase in inorganic P^{32}. Thus the assay was specific for ribonuclease activity.

The total ribonuclease activity in the homogenate and each fraction varied greatly depending on the pH. Mitochondria showed two distinct activity peaks both of about the same size at pH 5.8 and 7.8. Nuclei showed only one peak at 7.8. At this latter pH the specific activity of nuclei approached that of mitochondria. In the pH range 4.47 to 6.27 total activity expressed as per cent of homogenate activity was as follows: nuclei 10–17%; mitochondria 54–66%; supernatant 14–21%. In the pH range 6.8 to 7.8 the total activity of homogenate was from 20–50% of that at other pH values due probably to action of an inhibitor. Thus the activities of the fractions rose to: nuclei 90%, mitochondria 214% and supernatant 59% of homogenate activity; above pH 7.8 they dropped again. The small ribonuclease activity in nuclei is probably not due to contamination in view of the purity of the nuclei and the presence of only one activity peak.

Observations on the carbohydrate metabolism of the developing Chaetopterus egg. DWIGHT B. MCNAIR SCOTT.

A pattern of decrease of respiratory rate on fertilization and increase later was found agreeing with the findings of Whitaker. The mean rate before fertilization was $10\,\mu$ Mol O_2/hr./10^6 eggs, after fertilization $3.6\,\mu$ Mol O_2/hr./10^6 eggs, and about 5 hours later $12\,\mu$ Mol O_2/hr./10^6 eggs. The pattern of RQ values was similar to that reported by Ohman and others.

No reducing sugar was detectable in the eggs or the sea water in which they were suspended until about four hours after fertilization when reducing sugar appeared in both, only to disappear later. Glucose added to the sea water was taken up by the developing embryo at about the same rate as oxygen was consumed, after the blastula started to move.

Total carbohydrate was determined in the eggs by the anthrone method. The amount in unfertilized eggs was .015–.025 micrograms per egg, the total carbohydrate decreased in the unfertilized egg at the mean rate of $10\,\mu$ Mol/hr./10^6 eggs. After fertilization there was a sharp drop in total carbohydrate, followed by an increase. The blastulae contained a mean of .012 micrograms of total carbohydrate when they started to swim and also 10 hours later.

In 1951 S. S. Cohen found that enzymes to oxidize glucose-6-phosphate and 6-phosphogluconate could be extracted from Chaetopterus eggs both before and for twelve hours after fertilization. The measured activity of the extracts could account for the use of carbohydrate by these enzymes at the rate of 6–8 μ Mols/hr./10^6 eggs.

Thus it seems possible that carbohydrate utilized by the oxidative pathway may be the main substrate of respiratory activity in the unfertilized Chaetopterus egg.

The changes in carbohydrate after fertilization are more complicated, and no conclusions have been drawn from the experimental results.

Synthetic substrates for plasmin and thrombin. Walter Troll and Sol Sherry.

Plasmin and thrombin are usually classified as proteolytic enzymes. There seems to be little doubt that this classification is a fitting one for plasmin, an enzyme capable of splitting fibrin, fibrinogen and other proteins into peptide fragments. New evidence has been recently obtained which supports the proteolytic nature of thrombin's action. It has been shown that a peptide is split from fibrinogen by thrombin as the first step of the conversion of fibrinogen to fibrin. We have investigated esters of amino acids as possible substrates for these enzymes, and have observed that plasmin split esters of arginine and lysine, while thrombin attacked only arginine esters. The corresponding amides were also split, but to a much smaller extent. The rate of splitting of a typical substrate, such as tosylarginine methyl ester (TAMe), occurred with zero order kinetics as measured either colorimetrically by the disappearance of the Hestrine ester color, or titrimetrically by the appearance of carboxyl groups.

Three types of observations supported the view that these esters are substrates for these enzymes: 1. The proenzymes, plasminogen and prothrombin, showed no activity against these esters. Only on activation of the enzymes by known procedures did the activity appear. 2. Purification of the enzymes as measured by conventional methods led to a corresponding increase of activity against these esters. 3. The esters compete successfully with the protein substrates of the enzymes. Thus lysine ethyl ester and TAMe inhibit the proteolytic activity of plasmin on casein, and only TAMe inhibits the clotting of fibrinogen by thrombin. Other esters have no effect.

The fact that both plasmin and thrombin attack substrates of proteolytic enzymes confirms the proteolytic nature of these enzymes. Synthetic substrates offer a new quantitative tool for the measurement of these enzymes.

August 18, 1953

Studies on the mechanism of physiological dominance in Tubularia. Malcolm S. Steinberg.

In Tubularia very short pieces of the stem regenerate as partial bipolar hydranths, medium sized pieces (5–10 mm. long) regenerate a hydranth only at the distal end, and long pieces regenerate a hydranth at each end. Inhibition of proximal regeneration by distal regeneration is called "physiological dominance." It has been found that immediately after a stem-section is isolated, the entire cylindrical body (coenosarc) begins to stretch toward the distal end, depleting the proximal end of cells and supplementing the distal end. There being an effect of cell number upon rate of regeneration, this slows down regeneration at the proximal end, where it was already inherently slower (proximal regions normally regenerating more slowly than distal regions) and speeds up regeneration at the distal end. The distal regenerating hydranth, at about the time of its emergence, produces in active quantities an inhibitor which acts only upon regenerative processes operating before the hydranth primordium is established. The proximal end, due to its initially slower rate of regeneration and the further slowing down of that rate by the emigration of cells, is at this time in a pre-primordium stage and its regeneration is therefore completely prevented by the inhibitor. In very short stem pieces the distad movement of the coenosarc sets up a single primordium in the middle of the piece, this primordium containing all of the cells in the piece. If too few cells are present regulation of the primordium is imperfect and a partial bipolar form results. There is here no question of dominance, since that term refers to the suppression of one developmental center by another, and here there is only a single center. It is thought that proximal regeneration occurs in long stems because the inhibitor produced distally is diluted in the coelenteron to a sub-inhibiting level.

Elaboration of so-called posterior lobe hormones in the hypothalamus. Walther Hild.

Considerable evidence is presented that the vasopressor, antidiuretic and oxytocic hormones are not produced in the posterior pituitary. They originate by a neurosecretory process in the neurosecretory nerve cells in the supraoptic and paraventricular nuclei in the hypothalamus, and migrate in the nerve fibers of the supraoptico-hypophyseal tract into the neurohypophysis. Here they are stored and, if necessary, released. The product of the neurosecretory cells consists of a histologically stainable carrier substance, which contains the hormones and which can be separated from the hormones by solution in several organic fluids. This concept is based on the following observations:

1) The hormone content of the supraoptic and paraventricular nuclei, the pituitary stalk and the neurohypophysis—as shown by bioassays—depends directly on the amount of stainable material present in histological sections. The hypothalamo-hypophyseal system can be depleted of neurosecretory substances (carrier substance and hormones) by dehydration of the experimental animals. After the re-establishment of water balance, a re-accumulation of both fractions is observed.

2) After interruption of the supraoptico-hypophyseal tract (neurosecretory pathway) in dehydrated animals, carrier substance and hormones accumulate in the central fiber stumps and in the nerve cells, while the peripheral fiber stumps and the neurohypophysis remain empty.

3) Bioassays of the posterior pituitary tissue grown *in vitro* show that the neurohypophysis is unable to produce hormones as claimed by several authors. The hormones transferred with the explants into the culture medium are inactivated within 7 to 10 days, at which time the nerve endings degenerate and the stainable carrier substance disappears. Neurosecretory cells grown *in vitro* for from 12 to 68 days contain small amounts of stainable neurosecretory substance. Bioassays of these cultures are still under study.

GENERAL SCIENTIFIC MEETINGS

August 26–27, 1953

Abstracts in this section (including those of Lalor Fellowship Reports) are *arranged alphabetically by authors* under the headings "Papers Read," "Papers Read By Title" and "Lalor Fellowship Reports." Author and subject references will also be found in the regular volume index (published in the December issue).

PAPERS READ

The toxic effect of a marine bacterium on Limulus and the formation of blood clots.

F. B. Bang and J. L. Frost.

While injecting large Limulus crabs with various marine bacteria, a sick crab was discovered. Blood cultures, during a 6-day period, yielded a different bacterium which formed recognizably clear, translucent colonies. These consist of small gram-negative motile rods. Injection of large crabs with 0.1 cc. of a suspension of this bacterium caused death within 6–12 hours with concentrated suspensions (about 10^8 colonies/cc.) and produced persistent (5–6 day) active infections with lower dilutions. No active infections have been produced with relatively few bacteria. One-tenth cc. of boiled suspensions repeatedly killed 12-inch Limuli and in several experiments the supernatant from such boiled suspensions also killed.

A comparison of sizes of crabs (in which 2-inch to 12-inch individuals received the same dose of boiled suspension) showed that the large crabs are more susceptible to the toxic effect. This has not been shown for the active infections.

Within 10 minutes after the injection of the bacterial suspensions, the blood withdrawn fails to clot and has very few white cells. Later, evidence of white cell breakdown is found (cellular granules free in the serum and white cell processes identified in the electron microscope). The apparent intravascular clot is not in all cases irreversible and particularly smaller crabs will yield clear fluid during the first few hours after injection and then recover. The importance of this constant phenomenon in causing death is undetermined.

Preparations of Limulus blood for electron microscopy were made by bleeding crabs onto collodion coated grids and fixing with osmium at 3 and 30 minutes. Thorough washing before fixation was necessary to obtain clear pictures.

The blood cells so prepared showed numerous long processes (average width 54–96 millimicrons, extremes up to 320 millimicrons found) extending from one to another. These same preparations showed a network of minute fibers (average width 9–18 millimicrons, extremes up to 40 millimicrons found) extending between cells. Shadowed preparations showed no detectable periodicity.

A new type of respirometer which permits continuous and automatic recording of metabolic rate. FRANK A. BROWN, JR.

A respirometer is described with which it is possible to obtain continuous records of the fluctuations in rate of O_2-consumption of either undisturbed normal, or experimentally treated, organisms over periods up to several days. The method consists essentially of a respiratory chamber containing the organism and appropriate absorbents for CO_2 and any other significant gaseous wastes such as ammonia. Directly connected to this rigid chamber is a readily collapsible plastic (Visking Saran) sack whose lumen is continuous with the respiratory chamber by a fine capillary tube. The respiratory chamber is filled with air and the plastic sack with oxygen and the whole suspended by a thread in a constant temperature bath. The thread is made to activate an appropriately sensitive scale which records continuously the weight of the "diver" on a moving strip of paper. Each milliliter of oxygen consumed by the contained organism obviously increases the weight of the "diver" by one gram. The apparatus, in addition to having the virtue of being continuously recording, is one in which the organism is maintained in an environment of essentially constant oxygen for the duration of the runs. With such an apparatus as that described in principle here, it has been possible to demonstrate persistent diurnal, tidal, and semi-lunar rhythms of metabolism in the fiddler crab, Uca.

Colliding impulse studies of the relation between longitudinal transport and afterpotential in nerve of frog and squid. KENT M. CHAPMAN.

Recent authors have presented histological and chemical evidence for longitudinal migration of materials in living nerves, for neurosecretory activity, and for liberation of substances at nerve termination. A longitudinal ion displacement persisting during the afterpotential in stimulated nerve is predicted theoretically if one assumes that action potentials are propagated as waves (*i.e.,* with constant amplitude, shape, and conduction velocity) and that cellular fluids are ohmic media. Thus the transmembrane action potential, as the time integral of the longitudinal current, represents longitudinal charge (ion) displacement; hence afterpotential following excitation implies net ion transport. The calculated displacement for frog sciatic α fibers and for squid giant axons is 10^6 to 10^7 ions/ millivolt.

Experiments were designed to test for accumulation of this material at conduction blocks produced by collisions of pairs of impulses, using external non-polarizable electrodes to measure D.C. potential between the impulse collision site and some adjacent point on the nerve surface. With this technique, accumulating material can be detected only if it measurably alters the local membrane potential.

Colliding impulse potentials accompanying periods of repetitive thyration stimulation lasting 0.1 seconds to several minutes have been studied in desheathed bullfrog sciatics (α fibers) and in partially cleaned squid giant axons; 60 cycle stimulation was used for frog; 60, 120, 180, 240 and 300 cycles stimulation and mild veratrinization for squid. Spike amplitudes were normally 10–20 mv for frog, 15–40 mv for squid; negative afterpotentials were 0.5–1 mv for frog, 0.2–2.0 for veratrinized squid axon. However, in all cases, local changes in membrane potential at the collison sites were negligible; sensitivities of the measurements were 50–100 microvolts.

One must conclude that if the predicted ion accumulation takes place, the membrane potential is insensitive to the resulting qualitative or quantitative changes.

This work was supported in part by the Grass Trust.

Surface structure limitation of fertilization. RALPH HOLT CHENEY AND ALBERT I. LANSING.

The significance of physico-chemical characteristics of the egg surface to its fertilizability is an age-old problem. *Arbacia punctulata* has provided favorable material for study of the process of fertilization. Electron microscopical examination was used to compare the normal with caffeine-treated eggs. The latter results in inhibition of fertilization. Ultramicroscopic changes occur in the finer structure of the egg border and cortical granules as the egg is transformed from the fertilizable to the non-fertilizable state. The influence of the methylated xanthine, caffeine (1:3:7 trimethyl 2:6 dioxypurine), upon this sequence of events was determined. Unfertilized eggs from a mature female (egg sampling was 95% fertilizable) were shed into sea water as control, and into molarities of $M/5000$, $M/100$, and $M/10$ caffeine-in-sea-water for a 15-minute pretreatment prior to preparation for ultra-thin sections (0.1 mu to 0.25 mu) and examination under electronic magnifications of 3,000 to 10,000 ×. Enlargements photographically gave 15,000 to 35,000 ×.

The facts *that* $M/5000$ CSW is ineffective in exercising inhibitory effects upon the ability of the unfertilized egg to become fertilized in normal time and form, *that* $M/100$ CSW retarded the time and caused abnormalities in cleavage, *and that* $M/10$ CSW completely prevented fertilization, have been reported previously by Cheney. *The fact that* the normal Arbacia egg surface possesses numerous and fairly regularly-spaced papillae which are cytoplasmic prolongations was demonstrated by Lansing, Hillier and Rosenthal in 1952. This current study of the effects of these particular molarities of caffeine via electron microscopy reveals that an increasing degree of disorganization of the cortical granule matrix and a decreasing number in surface papillae occurs with increasing concentration of caffeine until the non-fertilizable $M/10$ egg cultures show a non-papillate, smooth surface.

Oscillographic analysis of an invertebrate equilibrium organ.[1] MELVIN J. COHEN.

A continuous discharge of impulses is observed on recording from the entire statocyst nerve in the lobster *Homarus americanus* Milne-Edwards. Such a spontaneous discharge is present with the preparation oriented in any position and is composed of action potentials from individual fibers firing aperiodically at a frequency usually less than 10 per second.

Rotation about the transverse axis results in a characteristic response pattern when recording from a specific nerve bundle. Rostrum-down displacement is accompanied by a burst of activity in the acceleratory phase, followed by a marked decrease (falling below the resting level) during deceleration associated with the termination of movement. Rostrum-up displacement produces an abrupt depression of activity during acceleration followed by a burst, which rises above the previous resting level, at the termination of movement. Impulse traffic returns to approximately the original resting level within one second after cessation of movement. The discharge rate also approaches the previous resting level 0.5 seconds after constant velocity is achieved and maintained following initial acceleration in either direction. Fibers of larger diameter (as judged by spike amplitude) cease firing with rostrum-up accelerations as low as 5 degrees per second per second, while the smaller fibers decrease in frequency only with greater accelerations and cease firing as values of 30 degrees per second per second are approached.

Single fiber preparations from other bundles of the statocyst nerve have been observed to maintain a non-adapting discharge in specific positions about the transverse axis. Such static "position" units most frequently attain a maximum of 20–30 impulses per second at 45–90 degrees rostrum-up and are often inactive in the zero (normal swimming) orientation. During movement toward the region of maximum static response these fibers may attain a frequency of 50 per second, adapting to the static level within 30–60 seconds after cessation of movement.

Effect of aqueous extract of beef spleen on cells of sarcoma 37 ascites tumor. IRENE COREY DILLER.

The injection of a single dose of aqueous beef spleen extract containing 100 mg./ml. of solid materials directly into the peritoneal cavity of mice bearing ascites sarcoma 37 resulted in inhibition of mitosis for a period of at least 48 hours, and in some cases in complete cessation of mitosis that persisted for even longer periods. Mitotic inhibition was accompanied by a marked inflammatory reaction. Cell counts per cc. of ascites fluid were lowered and the percentage of viable tumor cells decreased, partly as the result of cessation of mitosis and necrotic change, and partly through dilution with white blood cells.

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[1] Aided by a grant to Dr. Theodore H. Bullock from the National Institutes of Health.

Other samples of spleen extract produced similar changes at slower rates for the same dose level, but with a considerable increase in the total number of degenerating cells.

The reduction of tumor cell population was accompanied by the formation of islands of tumor cells which presumably served as centers for the formation of solid tumors. When the tumor masses thus formed were large, no regression was observed, though survival time was considerably lengthened; and small masses were completely resorbed under continued spleen extract therapy in 30.5% of the treated animals. No reduction in ascites swelling unaccompanied by formation of small solid tumors was observed.

Feeding efficiency of bats. EDWIN GOULD.

Since Griffin has suggested that bats may use high frequency sound to capture flying insects, the rate at which bats capture insects was studied by collecting 64 *Myotis lucifugus*, 5 *Pipistrellus subflavus* and one *Eptesicus fuscus* at night and weighing their stomach contents. Two hours after emergence from the roost a *Myotis* (body weight 7.6 grams) had accumulated 1.5 grams of insects. After 30 minutes flight two *Pipistrellus* contained 1.35 and 1.65 grams (25% of body weight) and one 18.9-gram *Eptesicus* caught 4.0 grams in 1.5 hours. Six *Myotis* which were collected after 45–95 minutes flight contained 0.9, 1.15, 0.91, 0.3, 0.05 and 0.95 grams. Even neglecting passage of food into the intestines this indicates a typical accumulation rate of one gram/hour under favorable conditions. Examination of 33 *Myotis* stomachs revealed remains of Lepidoptera, Coleoptera, Hymenoptera, and Diptera, but the great bulk of the material was too finely chewed for identification. Small Chironomidae (wingspread 3 mm., weight 0.2 mg.) were found, and bats were seen to pursue moths up to at least 20 mm. wingspread and 15 mg. weight. The hourly catch could thus be theoretically about 5000 Chironomidae or 66 moths, but an intermediate mixture is far more likely. Although individual moths are actively pursued it is possible that bats merely detect swarms of Chironomidae and fly through with their mouths open, employing a sort of "filter feeding." The volume of air "filtered," however, is only about 1800 liters per hour (assuming mouth area equals 1 cm^2. and flight velocity equals 5 meters/sec.), and most of the time is certainly not spent flying through dense swarms of insects. It thus seems equally possible that even individual insects as small as 3 mm. are selectively detected and pursued.

The effect of S^{35} on the development of Arbacia eggs. J. W. GREEN AND JAY S. ROTH.

Either dilute sperm or washed, unfertilized eggs of *Arbacia punctulata* were exposed to several concentrations of S^{35}. The S^{35} was prepared by evaporating carrier-free isotope solutions to dryness and taking up the S^{35} residue in sea water. The pH of such solutions was the same as that of sea water. Sperm were irradiated two hours in concentrations of 0.5, 5.0, and 50.0 microcuries of S^{35} per ml. prior to their use in the fertilization of non-irradiated eggs. Eggs were also exposed for zero, one and two hours to concentrations of 0.25, 2.5 and 25.0 microcuries of S^{35} per ml. prior to fertilization with non-irradiated sperm. After fertilization the time to 50% cleavage of the first division was determined, as well as subsequent developmental stage of the embryos between 48 and 72 hours. Irradiation of the eggs usually resulted in a small retardation in the first cleavage time with the intermediate and high concentration of S^{35} producing the longest retardations. The greatest amount of cleavage retardation occurred when eggs were fertilized and then irradiated; the least amount when the eggs were irradiated for two hours prior to fertilization. Roughly the reverse of this was observed in the subsequent development of the eggs. In some cases when control embryos were well developed plutei, the embryos in the highest isotope concentration were early gastrulae. Sperm irradiation retarded the first cleavage, even with 0.5 microcuries of S^{35}. Subsequent development of eggs fertilized with such sperm resulted in marked retardation of plutei formation, or in skeletal abnormalities.

The effect of calcium on the proteins of sea urchin egg homogenates.[1] PAUL R. GROSS.

Several significant events follow the addition of calcium to homogenates of sea urchin eggs (the eggs having been freed of Ca prior to homogenization). Among these phenomena are viscosity increase and rupture of cytoplasmic granules. The experiments reported here represent an attempt to understand these effects at the protein level.

In the presence of Ca, some of the protein becomes insoluble in water and in $\cdot M$ KCl. Study of this phenomenon over a wide range of concentrations of Ca strongly suggests that two reactions occur, one in the "physiological" range of [Ca^{++}], and the other when [Ca^{++}] is greater than 10 millimols/liter. Spectrophotometric measurements in the "physiological" range confirm a loss in protein solubility of ca. 20% and also indicate that the precipitated material is rich in nucleic acid.

By means of methods such as are used in the study of fibrinogen clotting, it is possible to demonstrate a small but consistent release of non-protein nitrogen as a result of the addition of Ca. This is consistent with previously reported proteolytic activity in the homogenates.

Electrophoresis of water and dilute salt-solution extracts of the homogenate reveals that, with low [Ca^{++}], only the slowest fraction is precipitated. Occasionally, a new, very slow boundary appears after Ca treatment.

The electrophoretic patterns are confirmed in electron micrographs of the insoluble parts of the homogenate. In the Ca-treated systems, aggregates appear. These are formed, apparently, by multipolar attractions, and are composed of spherical particles of diameter 256 ± 47 Å. Aggregates are absent from controls.

A simplified electrical method of determining the sex of sea urchins and other marine animals. ETHEL BROWNE HARVEY.

The electrical method of determining the sex of sea urchins described last year (Biol. Bull. 103: 284) has been simplified and improved. A household bell transformer (cost $1.50) is now used instead of a Variac to cut down the 110 A. C. voltage to 10 volts, the proper voltage for stimulating Arbacia. Lead electrodes have been found best; they are non-toxic and do not form bubbles as do platinum electrodes. These can be easily made from lead tubing having an outside diameter of 5 mm. with a bore of 3 mm. Short pieces of the lead tubing are placed around the ends of the copper wires from the transformer and sealed with tire tape and De Khotinsky cement to keep out the sea water. The free ends are flattened and sealed by pressure. Or short lengths of lead wire, 3 mm. diameter, in contact with the copper wire can be sealed with tire tape and De Khotinsky cement.

The two stimulating electrodes may be placed anywhere on the shell of the Arbacia, dorsal side up in sea water. Almost immediately after passing the current, the sperm or eggs exude from the five gonopores, the sperm in fine white threads and the eggs in small clumps. The emission of the sperm and eggs stops immediately when the current is cut off and begins again when the current is allowed to pass. The eggs should be removed at once to fresh sea water, and the sperm suspension kept in a refrigerator for further use. The eggs fertilize perfectly and develop normally.

By this method, males and females can be distinguished quickly with no trouble and without sacrificing an animal; and a small number of eggs can be obtained repeatedly from the same animal. The response is due to the stimulation of muscle cells in the walls of the ovary and testis. Animals containing only immature eggs do not respond.

Other animals also respond to the electrical stimulation, *e.g.* sand dollars, worms (M. J. Allen), molluscs. If a higher voltage seems to be required, a Variac should be used instead of the bell transformer.

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[1] This investigation was supported by a research grant from the National Cancer Institute, National Institutes of Health, Public Health Service, administered by L. V. Heilbrunn.

Evidence for independent mechanisms regulating potassium accumulation and sodium secretion in Ulva lactuca.[1] HUGH R. HAYWARD AND GEORGE T. SCOTT.

Investigations on factors controlling sodium and potassium distribution in cells of the marine green alga *Ulva lactuca* indicate the following. Cells washed in isotonic sucrose, having lost 80–90% of their normal K^+ and Na^+ will reaccumulate K^+ linearly when returned to sea water. The rate of reaccumulation is independent of the presence of Na^+ in the medium (isotonicity in the absence of Na^+ was maintained with sucrose), indicating that K^+ accumulation need not be secondary to Na^+ secretion. K^+ reaccumulation is complete. The metabolic inhibitor phenyl urethane (10^{-3} M) effects a marked progressive loss of K^+ and gain of Na^+, both of which changes are completely reversible on washing the alga in running sea water under illumination.

The presence of 5×10^{-3} M AsO_4^{---} completely protects the cells against the K^+ loss caused by 10^{-3} M iodoacetate (IA^-) in the dark, while it affords no significant protection against the Na^+ gain resulting from IA^- inhibition. This again indicates that separate mechanisms are operative in the regulation of Na^+ and K^+ concentrations in these cells. The K^+ protection by AsO_4^{---} is understandable in terms of arsenolysis at the level of triose phosphate dehydrogenase, the site of IA^- inhibition.

After 20 hours in the presence of 5×10^{-4} M IA^- (dark) samples were transferred to fresh sea water containing 5×10^{-3} M AsO_4^{---}; some were illuminated, the rest maintained in darkness. At the time of transfer the cell $[K^+]$ was 55% of the controls and the $[Na^+]$ ca. 150%. Continued marked loss of K^+ and gain of Na^+ occurred in the IA^- samples. The combination of AsO_4^{---} and illumination produced a slight reaccumulation of cellular K^+, while in darkness AsO_4^{---} offered the cells significant protection against K^+ loss as compared with the IA^- samples. The behaviour of the AsO_4^{---} samples as regards Na^+ differed markedly in light and dark: those in the dark continued to increase in cell $[Na^+]$, while those in the light secreted Na^+ to the control level within 12 hours.

These findings are consistent with the general association between carbohydrate metabolism and normal cation regulation. They further serve to emphasize two discreet mechanisms for controlling cellular $[Na^+]$ and $[K^+]$. Finally they may suggest an important relationship between the effects of illumination (in addition to generation of carbohydrate intermediates, and perhaps associated with the reductive powers of light) and the Na^+ secreting mechanism.

The effects of various acids on the receptors of the amphibian tongue. THOMAS N. JOHNSON. See abstract in "Lalor Fellowship Reports."

Adenosine triphosphate as an energy source in cell division; pressure-temperature experiments on the cleaving eggs of Arbacia and Chaetopterus. DOUGLAS MARSLAND,[2] JOSEPH LANDAU[3] AND ARTHUR ZIMMERMAN.

The eggs of these species, immersed in a .0005 M sea water solution of ATP, display a very definite improvement of furrowing capacity under widely varying conditions of temperature and pressure. At atmospheric pressure, the minimum temperature at which successful furrowing can occur is reduced by two degrees centigrade, and at higher temperatures, the minimum pressure required to block the cleavage furrows is increased by approximately 500 lbs. per square inch.

These increases in the cleavage potential appear to be mediated through the effects of the ATP upon the gelational state of the cortical cytoplasm of the eggs. Centrifugal measurements of the gel strength of the Arbacia egg cortex, made at 10°, 15°, 20°, 25° and 30° C., and at pressures ranging up to 8000 lbs./in., yielded values for the ATP treated eggs which were consistently 15–20% higher than for the control specimens. In both treated and untreated eggs the gel strength increased exponentially with rising temperature and decreased exponentially with rising pressure, the experimental and control curves being identical, aside from the higher position of the former.

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[1] Aided by a contract between the U. S. Atomic Energy Commission and Oberlin College.

[2] Supported by grant C807 from the National Cancer Institute.

[3] Damon Runyon Fellow, 1952–53.

The new data appear to provide further support for the cortical gelation theory of cytokinesis. Undoubtedly the cortical protoplasm of the egg represents a type II gel (Freundlich) in which the gelation process is an endothermic reaction; and tentatively it may be assumed that energy from the high potential phosphate bonds of ATP can be utilized for sustaining the gel structure of the dividing cell.

Integration in the cardiac ganglion of Homarus.[1] Donald M. Maynard.

By electrical stimulation, two cardio-accelerator fibers and one cardio-inhibitor fiber have been detected in the dorsal nerve of *Homarus*. The conduction velocity of the inhibitor is greater than that of the accelerators and is probably the largest of the three fibers which stain with methylene blue in the dorsal nerve.

Properly adjusted stimuli applied to portions of the isolated ganglion evoke a direct conducted response in only one fiber. This impulse in turn initiates activity in other units after a latent period of 0.02–0.04 seconds. This activity may be in the form and pattern of a "normal" burst, or it may consist of scattered impulses in a few units. The normal burst may be driven if the stimulus frequency is less than two per second and greater than the "spontaneous" rate of discharge. At higher frequencies, the burst may fail to follow each stimulus, coming after every other one. If a second stimulus is interposed in the latent period, the burst follows each paired stimulus at such frequencies. The latent period, which increases with increasing frequencies of stimulation, may also be shortened by the paired stimulus. Further facilitation is obtained with three or more stimuli, and driving may occur at still higher rates. The number of spikes in each burst is greatly reduced when the ganglion is so driven. If single stimuli are used at a frequency of 10 per second or more, the burst does not follow any particular stimulus, but the response rate increases with the frequency of stimulation; a stimulus frequency of 100 per second may produce an increase in burst rate of over 100%. When the unit responding directly to the stimulus is clearly a spontaneously active element, the burst often fails to follow as an entity at high frequencies and is disrupted as fibers selectively drop out with an increasing stimulus rate.

Isolation of midpieces from the sperm of Arbacia punctulata and observations on the role of the midpiece in sperm motility. Robert J. Neff.

Arbacia sperm midpieces are prepared by washing fresh sperm in 50% sea water, re-suspending in distilled water and removing clumped heads and tails by centrifuging 5 minutes at 4000 RPM in a Serval high speed centrifuge. Midpieces are concentrated from the supernatant by centrifuging 10 minutes at 8000 RPM. The midpieces may be washed once in distilled water. To avoid osmotic rupture, with release and clumping of mitochondria, further washes should be done in dilute salt solutions. Mitochondria of intact sperm, isolated midpieces, and ruptured midpieces take up Janus green from dilute solutions. Mitochondria of isolated midpieces or ruptured midpieces show intense succinic dehydrogenase reactions using the cytochemical neotetrazolium reaction.

Effects of dilute sea water on fresh sperm were studied by phase contrast microscopy. In 50% sea water all midpieces become displaced to one side of the head, giving "L" shaped sperm. The tails are always attached to the head, and perhaps the midpiece, at the outer tip of the "L". In 30% sea water, the heads first form crescents around the midpieces, then swell to rounded structures. Midpieces only begin swelling in 10–20% sea water, forming signet ringlike structures due to the two or more mitochondria attached to their periphery. In dilutions greater than 50%, the tail usually bends under with the midpiece and points in the same direction as the tip of the head. In 0.5 *M* KCl the midpiece and tail bend under, and eventually the tail forms a circle around the entire sperm. Movement is slowed in 30–40% sea water.

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[1] Aided by a grant from the Grass Trust for Research in Neurophysiology and in part by a grant to Theodore H. Bullock from the National Institutes of Health.

In favorable preparations, the midpiece is observed to execute rapid metronome-like movements in the absence of distal tail movement.

These observations indicate that *Arbacia* sperm is bilaterally symmetrical and suggest that most, if not all, of normal sperm movement originates in the midpiece with the tail acting as a sculling apparatus.

The motion of echinochrome granules in the unfertilized egg of Arbacia punctulata. ARTHUR K. PARPART.

In addition to a slight amount of Brownian motion the echinochrome granules (they are spheres ranging in diameter from 0.5 to 2μ) of unfertilized eggs of Arbacia also show a straight-line translatory motion which may amount to as much as 5μ per second at 24° C. This translatory motion is markedly slowed when the temperature is lowered to 10° C., while the Brownian motion appears unaffected. This is true of these granules whether near the surface or deep in the cytoplasm. Calcium ions added iososmotically to sea water do not affect this motion until the egg is killed by too high a concentration of calcium, at which point the whole cytoplasm gels. Sodium fluoride (0.01 *M*) and idoacetic acid (0.001 *M*) do not alter the translatory motion of these granules. The echinochrome granules of eggs whose cytoplasm has been partly separated by centrifuging do not show the translatory motion nor does recovery of the motion occur. These observations were made by T-V microscopy at 4500 × and recorded by motion pictures. It is suggested that the echinochrome granule may have several temporary and contractile fibers attaching it to the surface of the egg and the shortening of these fibers may lead to their zigzag motion thru the cytoplasm. The translatory motion of these granules is frequently across the stream of the marked cytoplasmic streaming evident in the unfertilized egg.

Cation interrelationships at the Fundulus ectoderm. D. R. SHANKLIN.

Using dechorionated 5–7 day *Fundulus heteroclitus* embryos as the living material, cation relationships were studied with the radiotracer elements: Na^{22}, Na^{24}, K^{42}, and Ca^{45}. The solutions, made to conform to an analysis of the Woods Hole supply sea water, contained these ions and magnesium in monary, binary, ternary, and quaternary combinations, with chloride as the sole anion. The exchangeable ions were found to have been equilibrated by ten hours; thus this was chosen as the end point. The major findings are: (1) In binary states, with the uptake from monary media as the base, calcium and sodium are mutually inhibitive; potassium and calcium are mutually inhibitive; sodium facilitates potassium, and potassium inhibits sodium; magnesium inhibits sodium and potassium, and facilitates calcium. (2) In ternary mixtures, *e.g.*, CaNaK, all three are inhibited, in a degree predictable by the summation of binary effects. (3) Magnesium plays an exotic role, for the union of both bivalent ions with either univalent ion is distinctly favorable to the uptake of any of the three tracer elements, when compared to the union of either bivalent ion separately, including the binary solution CaMg. These effects of magnesium are not additively predictable from binary action. (4) The complementary consideration of absence of an ion yields a corresponding picture. (5) In summation effects to the quaternary state, the effects are additive for sodium and calcium, but not for potassium; indeed, potassium is singularly facilitated above that predictable from binary and ternary actions. (6) Sea water was run correlative to the quaternary solution, and anion effects, facilitative for sodium and potassium, and inhibitive for calcium, were found. (7) The activity of the ions in these media is in reverse order of their concentrations, which is $Na > Mg > K > Ca$, the activity of magnesium a predicted value from the data.

The effect of rapid shifts of sodium and potassium on the membrane potential of muscle fibers of the spider crab, Libinia emarginata. WILLIAM K. STEPHENSON.

If leg muscles of the spider crab, *Libinia emarginata*, are isolated in sea water, the fibers loose K^+ and take up Na^+ and Cl^- at a rapid rate. From original concentrations of 93 mM./Kg. wet weight (mM.) for Na^+ and 115 mM. for K^+, the cation balance shifts to about 250 mM. of Na^+ and 50 mM. of K^+ within 1 hr.; each process having a half-time of 15 minutes.

Chloride increases somewhat more slowly from 80 mM. to 160 mM., with a half-time of 20 minutes. Muscle preparations do not change weight during the first 30 minutes of soaking in sea water, and during the next 90 minutes gain only 10% over their original wet weights. Thus, the large increase in Na^+ and Cl^- concentrations is not primarily due to a loss of water to the hypertonic medium.

Using the glass micro-electrode technique of Ling and Gerard, a membrane potential of 69.2 ± 1.3 (S.E.) mV. was obtained for muscle fiber preparations isolated in sea water. A study of the time course of potential change following immersion of the muscle in sea water reveals that the potential rises to a constant level within 10 minutes and thereafter does not change appreciably for two hours. Crab muscle, therefore, maintains a constant potential while very large shifts of Na^+, K^+, and Cl^- are taking place across the fiber surface.

In cases of rapid ion movement, the membrane potential is independent of any simple ion gradient, and thus the familiar Nernst equation does not apply. Further, the mechanism responsible for maintaining the membrane potential is effective in the face of a high permeability to Na^+ and K^+, and must be operating with a large enough safety factor to prevent these ions from "shorting out" the potential as they diffuse inward in response to their electrochemical gradients.

(This work was aided in part by Contract Nonr–24900 Office of Naval Research administrated by H. B. Steinbach.)

PAPERS READ BY TITLE

Osmoregulating capacity in populations occurring in different salinities. John D. Anderson and C. L. Prosser.

Populations of blue crabs and quahogs from different salinities were tested for differences in osmoregulation. Osmoconcentration was measured by a melting point method and expressed in equivalent normality of NaCl. Average normal concentrations at low tide collections are:

	Location	Water osmoconcentration	Blood osmoconcentration
Crabs	Pettaquamscutt River		
	Near mouth	0.43 (N NaCl)	0.46 (N NaCl)
	Head of lake	0.10	0.38
Quahogs	Great Pond, Falmouth		
	Near mouth	0.40	0.44
	Perch Pond	0.20	0.33

Animals from high and low salinities were put under stress by placing them directly into a series of dilutions of sea water. Survival in concentrations lower than 10% S. W. was significantly greater for crabs from low than from high salinity. The crab blood concentration was regulated over a wide range of external concentration, but regulation failed in high salinity crabs at about 0.1 N NaCl equivalent and in low salinity crabs at 0.04 N NaCl equivalent. In 100% S. W. (0.51 N NaCl equivalent) blood was hypotonic (0.46 N NaCl). After a week in 100% S. W. the blood concentrations and critical external concentrations for both groups were similar. In contrast to Callinectes, quahogs and marsh mussels adjust their blood to external concentration as long as they pump water but Venus failed to pump below about 50% S. W. and Modiolus failed below 35% S. W. Quahogs from dilute regions tended to pump at lower concentrations than from concentrated water. It appears that natural populations of Callinectes and Venus from different minimal salinities differ in state of osmotic adaptation; stress tests applied thus far indicate these differences as phenotypic.

On the hydrolysis and transpeptidation of glutathione in marine forms. Eric G. Ball, Octavia Cooper and Edith C. Clarke.

A manometric method has been developed to follow the reactions whereby glutathione undergoes either enzymatic hydrolysis or transpeptidation at its γ-glutamyl peptide linkage. It is based upon the more marked ability of one product of these reactions, cysteinylglycine, to coordinate with ferrous iron and carbon monoxide than is shown by glutathione itself. The reactions are carried out in an atmosphere of CO at 30° C. and pH 9.0. The various tissues studied have been homogenized usually in borate buffer. Fish kidney shows the most activity of all marine tissues studied. Results are expressed in μmols of CO uptake per 100 mg. of tissue per hour. (Theoretically 1 μmol of CO is taken up for each μmol of glutathione reacting.) Values for the kidney of the following species are as follows: scup, 3.51; sea bass, 2.51; tautog, 1.97; dogfish, 1.70; butterfish, 1.39; toadfish, 0.53. In contrast to mammalian kidney preparations (which are 10–20 times as active), no significant difference in rate is observed in the presence or absence of alanine. Whether this indicates absence of transpeptidation in fish kidneys remains to be determined. The spleen of all these fishes shows a low activity (0.37–0.03). The heart, brain, liver, gonads, gills, intestine and swim bladder red gland of fish show no activity. No activity could be found in the eggs and sperm of *Arbacia punctulata* or various organs of *Venus mercenaria* and *Busycon canaliculatum*. The distribution pattern of the enzyme is thus not consistent with the hypothesis that it is involved in protein synthesis. If the enzyme is important to kidney function, then the assignment to it of a role in absorptive processes seems favored by a consideration of the relation of enzyme content to the structure and function of the kidneys studied (*e.g.*, toad fish).

A further study of the effects of centrifugation and low temperature on the development of Fundulus heteroclitus.[1] LOY J. BARNES.

It has previously been shown that centrifugation of *Fundulus* eggs shortly after fertilization at low forces of gravity produces total or partial duplications in a low percentage of cases. The present experiments consisted in varying the time of treatment and the force of gravity employed. Eggs were handled as previously described. Centrifugation of eggs beginning from 10 to 90 minutes after fertilization confirmed previous observations that a "sensitive period" for the production of duplications occurs within the first 30 minutes after fertilization when the blastodisc is forming. Doubling the force previously used (to ca. 600 g.) did not produce significant increase in the occurrence of duplications. A combination of centrifugation (at 300 and 600 g.) and low temperature (4° C.), the latter applied during the first 24 hours after fertilization, did not produce duplications in higher frequency than occurred in some batches of eggs which were only centrifuged. No general conclusions can be drawn as to a method for producing duplication by altering gravitational force at this time, but the types of abnormalities found (in addition to true duplication), including reduced and defective head structures, mesodermal deficiencies and rudimentary "parasitic twin" structures, suggest that any agent capable of separating a presumptive organization center produces effects commensurate with the degree of redistribution of ooplasmic materials.

Elastic and ionic studies with the squid giant axone. L. L. BOYARSKY.

The static elastic modulus of cleaned squid axones was determined tensiometrically. The order of magnitude of Young's modulus was 10^9 dynes/cm.^{2} This value is attributed to the connective tissue components since crushing a small central region does not affect the stress-strain curve. The breaking strength of normal axones was 4.9 ± 0.7 grams. The magnitude of the action potential obtained with fixed external electrodes was independent of stress up to break. Yield was not plastic and occurred at strains of about 30%. Conduction time and rate of rise of spike were also unchanged up to these extensions. Similar studies were carried out with nerves placed in artificial sea waters. Control studies showed that with unstretched nerve at *in situ* lengths, magnesium will substitute for calcium in repressing spontaneous activity. Nerves survived equally well at 50 mM calcium or magnesium concentrations, the other constituents being at the usual concentrations found in sea water. At 15–20 mM calcium or magnesium, the other ion having been excluded, spontaneous activity began. Nerves survived for hours at these concentrations. The magnitude or rise time of nerves kept in such low-calcium solutions was unaffected by stretch. These results suggest the presence of isolated areas in the membrane which permit ionic transfer during propagation. During stretch these areas are unaltered.

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[1] Supported by funds from U. S. Public Health Service and the Office of Naval Research.

Diffusion coefficient of hemerythrin. ARTHUR S. BRILL AND JOHN M. OLSON.

Three-tenths per cent solutions of hemerythrin from the marine annelid, *Phascolosoma gouldii,* were prepared in 0.1 *M* phosphate buffer of pH 7.0. Diffusion of hemerythrin from solution into solvent was recorded in a Perkin-Elmer portable Tiselius electrophoresis apparatus.

By the Longsworth scanning method, two oxyhemerythrin boundaries at 22° C. were photographed three times in a period of 36 hours. During this period a gradual transition to methemerythrin was observed. Similarly two methemerythrin boundaries at 20° C. were photographed three times in a period of 20 hours. In this case the solutions had become noticeably cloudy by the end of the observation period.

For each boundary a diffusion coefficient value was calculated by the maximum ordinate-area method. All values were converted to the standard conditions of $t = 20°$ C. and solvent viscosity equal to the viscosity of water. The values from the oxyhemerythrin boundaries are 5.90×10^{-7} and 5.27×10^{-7} cm.^{2}/sec. The values from the methemerythrin boundaries are 5.87×10^{-7} and 6.27×10^{-7} cm.^{2}/sec. The average of the four values is 5.8×10^{-7} cm.^{2}/sec.

Temperature independence of the frequency of the endogenous tidal rhythmicity of the fiddler crab, Uca pugnax. FRANK A. BROWN, JR., MIRIAM F. BENNETT AND MURIEL I. SANDEEN.

The influence of temperature upon the physiological mechanism determining the frequency of the tidal rhythm was investigated. This was done by utilizing the fact that the average stage of the melanophores in legs isolated from normal *Uca pugnax* one-half hour following their autotomy reflects directly the tidal rhythm within the animal by being at a minimum in legs removed at the time of day of high tide in the region of collection of the animals. A large number of crabs was divided into three lots two to three hours preceding the time of high tide. One lot was maintained in sea water at 30–32° C., a second lot at 12–14° C., and the third lot at room temperature. Legs were caused to be autotomized from animals removed from each of the three lots at hourly intervals over a 5- or 6-hour period. The fact that the three lots arrived synchronously at the condition of minimum dispersion in the isolated legs, coinciding accurately with the actual time of high tide, clearly demonstrated that there was no influence of temperature upon the frequency of the tidal rhythm over the 18 to 20° temperature range thus investigated.

Endogenously regulated diurnal and tidal rhythms in metabolic rate in Uca pugnax. FRANK A. BROWN, JR., MIRIAM F. BENNETT AND H. MARGUERITE WEBB.

The O_2-consumption of normal *Uca pugnax* was determined continuously over a lunar period in automatic, continuous-recording respirometers. The crabs were collected at intervals and several run concurrently in individual respirometers at constant temperature and illumination for 2 to 4 days each. The daily respiratory-rate pattern varied systematically from day to day, clearly exhibiting evidences of persistent diurnal and tidal rhythms. The form of the diurnal rhythm was ascertained by averaging the respiratory rate for each hour of the day for a whole 15-day tidal period. The tidal rhythm was thereby randomized in that all phases of a tidal cycle occurred at each time of day. The diurnal cycle exhibited increasing metabolic rate from midnight to about 6 A.M., decreasing to a minimum about noon, then increasing to a second maximum about 10 P.M., with a second but lesser minimum occurring about midnight. Moving consecutive daily respiratory patterns backwards at an average rate of about 50 minutes per day, thereby keeping the tidal cycles in phase and utilizing in this manner 29 days of data, the diurnal rhythm was randomized, and a clear tidal rhythm of metabolic rate became evident. The maximum O_2-consumption occurred about low tide, and minimum rate about high tide. The overall average rate of O_2-consumption was about 50 ml./kg./hr. and in both the diurnal and the tidal cycles, an average of about a 60% increase was involved in passing from minimum to maximum rates.

An antimitotic substance in the ovary of the common puffer, Sphaeroides maculatus.[1] PIERRE COUILLARD.

The ovaries of the starfish and of other invertebrates contain antimitotic substances which apparently owe their activity to their heparin-like nature (Heilbrunn, Wilson and Harding, 1951). Among the vertebrates, fishes of the family Tetraodontidae contain in their ripe ovaries a poison, tetrodotoxin, whose empirical formula, molecular size and action on the frog heart suggest a relation to the breakdown product of heparin found last year by Chaet. Accordingly, an attempt was made to discover if ovary extracts from a local tetrodont fish (*Sphaeroides maculatus*) had an antimitotic action and if, moreover, the physiological effects of such an extract could be related to the presence of heparin-like substances. Unfortunately only spent ovaries were available; they contain little or no tetrodotoxin.

Neutral sea-water extracts of such ovaries will, in low concentration, induce abortive cleavage of Chaetopterus eggs previously fertilized in sea-water; a well-defined, almost equational furrow forms which soon regresses. Phase contrast shows, however, that karyokinesis proceeds normally in these eggs as an increasing number of amphiasters can be observed. If, from threshold concentrations of the extract, the eggs are transferred into sea-water, they divide almost at once into a corresponding number of blastomeres. Higher concentrations completely and irreversibly inhibit cleavage; centrifugation experiments show that in these cases, mitotic gelation is prevented. The extracts have little or no effect on the clam heart. Preliminary experiments indicate that the active principle of the extracts is not readily dialyzable.

Acid hydrolysates of the ovary contain considerable hexosamine (about 2 mg. per gm. of fresh tissue); extraction by the phenol partition technique (for heparin), yielded a metachromatic, anticoagulent substance; this presumed heparin has accounted so far, for 30% of the total hexosamine.

Thus it seems that, besides tetrodotoxin (still to be investigated), the puffer ovary contains a large heparin-like molecule, presumably responsible for the antimitotic action of ovarian extracts.

Clotting of the perivisceral fluid of the sand dollar, Echinarachnius parma. ERIC DAVIDSON.

Clotting occurs after tissue injury. The clot is formed exclusively from cellular elements; the extracellular fluid does not clot. Cells separated from the plasma by centrifugation and re-suspended alone in sea water will aggregate to form a clot, though slowly. The separated plasma does not gel.

Red and white amebocytes are present in the perivisceral fluid. Red cells do not break down, lose color, or shape, in a clot; white cells are the clotting agents. In clotting, the exterior processes of the white cells contract to form fibers, which interlock with those of other cells.

No pigment granule breakdown (such as occurs in Arbacia) accompanies clotting.

The tendency of the perivisceral fluid to clot around foreign particles (such as fine washed sand) can be utilized in establishing an end point in determination of clotting time.

When tissues are injured, a tissue factor is released which is essential for the clotting process. Besides this tissue factor, free calcium is also necessary for clotting. Calcium is involved in white cell breakdown. After white cell breakdown, calcium absence does not inhibit clot formation, but previous removal of calcium prevents breakdown and clotting; 0.35 *M* Na citrate was used to remove free calcium.

Tissue factor is released in the absence of calcium though it is then ineffective. If tissue factor is absent, then calcium alone cannot effectively induce clotting. Cells separated from tissue factor in the plasma by centrifugation clot eventually; either because they secrete tissue factor or because the original tissue factor is present, adsorbed to the surfaces of the cells. Such clots are abnormally thin.

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[1] This investigation was supported by a research grant from the National Cancer Institute, National Institutes of Health, Public Health Service, administered by L. V. Heilbrunn.

Some properties of the mitotic inhibitor in starfish ovary extracts.[1] ARNOLD DUNN.

Heilbrunn, Wilson and Harding (1951) showed that acid sea water extracts of starfish ovary can prevent cleavage in marine eggs and that this inhibition is accompanied by a liquefaction of the cytoplasm of these eggs.

Heilbrunn, Chaet, Dunn and Wilson (1952) reported that after ammonium sulfate fractionation of the extract, the active substance remains with the globulin fraction. If extracts were placed in cellulose sacs and dialyzed against sea water, the active substance passed through the sac. Since the activity of the antimitotic substance was destroyed by periodate and since crude extracts were metachromatic when tested with toluidine blue, the indication was that the antimitotic substance might be a polysaccharide similar to heparin.

If a solution of the globulin fraction is dialyzed against 0.3 N sodium chloride for 48 hours or longer and the dialysate chromatogrammed on paper, the chromatogram shows the presence of carbohydrate and the absence of proteins, amino acids, and nucleic acids. The dialysate is metachromatic; this metachromasia disappears when globulin proteins are added. Apparently the active substance may be combined with protein as a mucoprotein which can be disassociated by dialysis. To test this hypothesis, proteins of the crude extract were separated by paper electrophoresis. Protein polysaccharide complexes were detected by the method of Köiw and Grönwall. These tests show that polysaccharides migrate with the globulin peak. If the globulin fraction is digested with trypsin and the resulting suspension treated to remove trypsin and remaining proteins, polysaccharide can be precipitated with alcohol. This polysaccharide prevents cell division in Chaetopterus eggs. The inhibition can be reversed by returning the eggs to sea water. The polysaccharide also prevents the clotting of sheep plasma and is metachromatic. Thus it seems clear that the antimitotic substance is similar to heparin; within the cell it may be bound to globulin proteins.

Effects of X-radiation on oxidative systems of Tetrahymena pyriformis. HERBERT J. EICHEL AND JAY S. ROTH.

Tetrahymena pyriformis S. grown in proteose-peptone for 72 hours, were used to prepare cell concentrates or cell-free homogenates. Cell respiration, and the activity of several enzymes in the homogenates were determined after irradiation with 300,000 or 600,000 r. Succinic (1), malic (2), and glutamic (3) dehydrogenases were assayed manometrically and catalase (4) titrametrically in homogenates prepared from irradiated cells and in homogenates which were irradiated directly. With the latter, the following changes were observed at the end of 80 minutes post-irradiation: at 300,000 r, (1) −40%; at 600,000 r, (1) −45, (2) −50, and (3) −20%. Immediately after irradiating homogenates (4) decreased 25%, while 1–2 hours later the activity increased to that of controls. After 24 hours, (4) was 50% more active in the irradiated homogenates than in the controls. With cells irradiated at 300,000 r and then homogenized, (1) decreased 9% 80–140 minutes following exposure. In homogenates prepared from cells irradiated at 600,000 r, (4) was unchanged 2 hours after exposure.

At 300,000 r respiration of concentrated cell suspensions was only slightly affected, but at 600,000 r it was decreased by 17, 40, and 53% at 30, 80, and 140 minutes, respectively, after irradiation. 1,2-transcyclopentanedicarboxylic acid, and 2,6-diaminopurine, which have been reported to alter cell permeability, protected irradiated cells under some conditions. The oxidation of L-phenylalanine and acetate by irradiated and control cells was also studied. Oxidation of phenylalanine by irradiated cells was depressed but irradiated organisms showed greatly increased oxidation of acetate. Thus, with 300,000 r at 30, 80, and 140 minutes post-irradiation, respiration of irradiated cells diluted 1:1 with distilled water was depressed by 34, 38, and 43%, respectively, while with irradiated cells added to acetate the corresponding changes were +2, −13, and −32%. As a result of the increase in acetate oxidation by irradiated cells, the net effect was a significant protective action on respiration achieved by treatment *after* irradiation, one of the few examples of such action.

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[1] This investigation was supported by a research grant from the National Cancer Institute, National Institutes of Health, Public Health Service, administered by L. V. Heilbrunn.

A study of some proteolytic factors in the unfertilized Chaetopterus egg.[1] LESTER GOLDSTEIN AND NORMA O. GOLDSTEIN.

In a recent paper (Goldstein, 1953) it was postulated that a protease was involved in the breakdown of the germinal vesicle nucleus of the egg of *Chaetopterus pergamentaceus*. Consequently, an attempt was made to isolate such an enzyme from unfertilized eggs, and to study its behavior and the relationship of such behavior to the dissolution of the nuclear membrane.

Generally, the eggs in sea water were freeze-dried, the resultant powder re-suspended in 0.035 M sodium citrate, and the suspension homogenized. These preparations were tested for proteolytic activity by a modification of the Anson technique.

Apparently there are at least two different proteolytic factors present. One, with a relatively high activity, has a pH optimum of approximately 2.8 if a hemoglobin substrate for peptic digestion (Anson) is used; with a hemoglobin substrate for tryptic digestion (Anson) the pH optimum is approximately 4.0. The other factor, which has relatively low activity, apparently has a broad pH optimum, about pH 7–9, and appears to behave differently with each of the 4 substrates employed (the two mentioned above, with and without Ca).

In the paper mentioned above it was suggested that the protease involved in nuclear breakdown would show a rise in activity following liberation of the eggs from the ovary, and that after the breakdown had occurred the activity would fall off—as a result of some protective "brake" mechanism. Analyses of homogenates prepared at various times after the eggs were shed indicate that only the protease with the acid pH maximum (especially when tested on substrates prepared for tryptic digestion) behaves in the postulated manner—the other protease does not. The picture is not perfect because adequate data could not be obtained on the early stages, *i.e.*, immediately following liberation of the eggs.

Photosynthetic activity of phycobilans in some red and blue-green algae. F. T. HAXO AND PHYLLIS S. NORRIS.

Photosynthetic action spectra of some Woods Hole algae have been determined over the past several summers, employing the polarographic method for oxygen determination applied by Haxo and Blinks to Pacific Coast marine algae.

Photosynthetic activity in the bright red, deep-growing red alga, *Grinnellia americana*, follows closely thallus absorption in the middle portion of the visible spectrum where absorption is due almost exclusively to r-phycoerythrin, but deviates downward from thallus absorption in the red and blue portions of the spectrum where chlorophyll *a* absorbs strongly.

Vegetative portions of *Porphyra umbilicalus* thalli (olivaceous in color) display action and absorption spectra rather like those reported earlier for the West Coast species, *P. perforata*. Somewhat similar results but with evidence of greater phycocyanin activity have been obtained for the bluish-green colored, fresh-water red alga, *Batrachospermum sirodotii*. The absorption spectrum of the yellowish male margins of *P. umbilicalus* resembles that of green algae, the absorption being much higher at 675 mμ than at 560 mμ, apparently due to a considerably decreased phycerythrin/chlorophyll ratio. The corresponding curve for photosynthetic activity showed a definite, but minor, peak at 675 mμ; however, maximum activity was in green light.

Action spectra of *Porphyridium cruentum* (from cultures), a red alga which contains a single peaked phycoerythrin, showed maximum activity in green light and very little activity in regions of strong chlorophyll absorption. Similar results were obtained with *Phormidium ectocarpii*, a red-colored, marine blue-green alga, isolated in unialgal culture at Woods Hole.

In general these results confirm and extend earlier reports that the phycochromoproteins are the major active light absorbers in the photosynthesis of the Rhodophyta (and possibly the Cyanophyta), the light absorbed by chlorophyll *a* being utilized with relatively lower efficiency.

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[1] This investigation was supported in part by a research grant from the National Cancer Institute, National Institutes of Health, Public Health Service, administered by L. V. Heilbrunn.

The action of parthenogenetic agents on the interior protoplasm of the Chaetopterus egg.[1] L. V. HEILBRUNN AND WALTER L. WILSON.

For some time now it has been our belief that stimulation in general, and stimulation to mitosis in particular, involve a release of calcium from the outer cortex of the cell and a clotting or gelation in the cell interior. Evidence for the first part of this thesis was presented last year (Wilson and Heilbrunn, 1952) for the egg of the marine worm Chaetopterus. Using the same egg, we have now been able to show that all the agents that incite the cell to divide do actually cause a sharp increase in the viscosity of the interior protoplasm. We have studied the effect of hypertonic solutions, of acid and alkali, of isotonic potassium chloride solutions, of ultraviolet radiation, and of heat and cold. All these agents induce cell division; and not infrequently the treated eggs go on to produce motile larvae. During or after exposure, tests of protoplasmic viscosity were made with the centrifuge method. In all cases, exposures which cause the egg cells to divide also produce a gelation in the protoplasm. Moreover, there is a rather close correlation between the percentage of cleavage produced and the percentage of eggs showing gelation. If, however, the gelation is too intense, death rather than cell division may ensue.

The results provide additional evidence for the calcium release theory of excitation, and on the basis of this theory it is possible to interpret the action of the many diverse agents that induce cells to divide.

Antimitotic substances present in ovaries.[1] L. V. HEILBRUNN, WALTER L. WILSON AND MURIEL LIPPMAN.

In many species of animals, eggs remain for long times in the ovary without undergoing mitotic division; then as soon as they leave the ovary, they divide. This is an indication that antimitotic substances may be present in ovaries and indeed our earlier work has shown that the starfish ovary contains a very potent antimitotic substance and that such substances are also found in the ovaries of clams and sea urchins. In the present study, we have extracted the ovaries of various animals with acidified sea water, then neutralized the extract and tested its effect on cell division in the egg of the worm Chaetopterus. We have found antimitotic substances in the ovaries of a wide variety of animals, including the lobster, 7 species of fishes, a frog, a salamander, and 3 species of mammals. The potency of these antimitotic substances varies widely, but all of them act like starfish ovary extract in keeping the protoplasm fluid and preventing mitotic gelation. It must not be thought that the ovary is unique in containing antimitotic substances; indeed we believe that it is a characteristic of all types of cells to contain heparin-like antimitotic substances. However, in general, whenever we compared the potency of an ovarian extract with the potency of an extract from some other organ or tissue, as for example liver or muscle, we found that the extracts obtained from the ovary were more potent in their antimitotic effect.

Use of autotomized legs in determining the phases of the tidal rhythm in Uca pugnax. MARGARET N. HINES.

Earlier methods of determining the phases of the endogenous tidal rhythm of the fiddler crab have involved the determination of the phases of the 15-day rhythm which results from the simultaneous possession of both persistent diurnal and tidal rhythms. The method to be described enables the phase to be determined in a few hours of a single day. To do this, the crabs are kept in pans in the laboratory and at intervals through the day sample animals are induced to autotomize their legs. The melanophores of the legs at the time of their isolation typically have their pigment dispersed. Following isolation the pigment tends to concentrate.

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[1] This investigation was supported by a research grant from the National Cancer Institute, National Institutes of Health, Public Health Service, administered by L. V. Heilbrunn.

The chromatophore stage observed at 30 minutes has been found to vary in such a manner that the stage is at a minimum at the time of day of high tide in the place where the animals had been collected.

Physiological studies of centrifuged normal and carcinogen-treated Tetrahymena. MIRIAM I. JACOB.

Tetrahymena pyriformis (strain E) were serially transferred for more than 600 generations through media containing various carcinogens in 2% proteose peptone, 0.2% yeast extract, and 1% polyoxyethylene-40-sorbitol cottonseed oil (Atlas Compound G-1394). The carcinogens were dissolved in G-1394 and then added to proteose peptone media. Addition of the solubilizer to the media caused a significant increase in the period of logarithmic growth and in the total number of organisms, compared to a control medium. Growth rate and number of organisms was considerably reduced in media containing 100 μ gm./ml. of 4-aminoazobenzene, 4-monomethylaminoazobenzene, or 4-dimethylaminoazobenzene. Serial transfers for about 300 generations through 4-aminoazobenzene and 4-monomethylaminoazobenzene caused decrease in size, and alteration in shape, of *Tetrahymena*. Organisms grown in azo dyes contained granules and globules which were yellow, compared to similar, colorless, organelles in control *Tetrahymena*. Centrifugation at 7000 g for 90 minutes in a gradient medium composed of 20% gum arabic and distilled water resulted in elongation, stratification, and fragmenting of the cells into light and heavy halves. *Tetrahymena* grown in proteose peptone have a higher density than organisms grown in media containing G-1394, with or without carcinogens. Cells from 7 day old cultures, although still in the log phase of growth were more prone to fragment than those from 72 hour cultures. Fixation and staining of centrifuged *Tetrahymena* indicated the following distribution of intracellular components. The nucleus was displaced to the heavy half. The light half was filled with granules and globules which occurred in larger numbers in media containing G-1394, then in control proteose peptone medium. Treatment with OsO_4 indicated that lipid material was concentrated in the light half. The light half of stratified whole cells grown in media with azo dyes was bright yellow. This suggests that the azo dyes are associated with the lipoidal components of the cell.

Structure of the axoplasm as revealed by spontaneously-formed vesicles. CHIEN-YUAN KAO.

In freshly isolated giant axons of the squid, there are numerous well-formed vesicles in the peripheral layer of the axoplasm. These vesicles are oval, about 30 μ in width and 50–150 μ in length, tending to occur in chains of four to six individual components or in conglomerates. They are formed from filamentous structures which are visible within a few minutes after dissection. Nodose swellings appear in these filaments which gradually enlarge and fuse into distinct oval vesicles. The vesicles are transparent and have a faint pink tinge. They are structureless except for an investing film which has a slightly higher refractive index than the axoplasm.

When the axons remain in the gel state, the long axes of the filamentous structures and the vesicles are always arranged parallel to the long axis of the nerve fiber. In this state, the vesicles do not float upwards in vertically placed nerves, nor can they be moved about by a micropipette. When the axoplasm becomes a sol after the nerve has been kept in sea water, or as the result of injections of divalent cations Ca^{++}, Mg^{++}, Sr^{++}, and Ba^{++}, these vesicles round up into spheres and can be moved about by the tip of a micropipette or a micro-stream of injected fluid. These observations confirm previous studies with micro-injected oil drops that there is a linear organization in the fresh axoplasm and that the divalent cations have an unusual liquifying effect on axoplasm, in contradistinction to their coagulating action on other types of protoplasm.

The vesicles maintain their form in axoplasm extruded from the nerve, but disappear on treatment with alcohol, probably because the investing film is alcohol-soluble. The formation by progressive swelling, the slightly higher refractive index and the alcohol solubility of the investing film suggest that the vesicles may represent intra-axonal myelin forms comparable to those observed in electron micrographs of vertebrate nerves.

The effect of a sulfide-blocking reagent on the oxygenation of hemerythrin. I. M. Klotz, S. Rapaport and E. V. H. Rosenberg.

It has been shown by Riggs that *p*-chloromercuribenzoate affects the interactions among the heme groups of hemoglobin but does not change the total uptake of oxygen. Markedly different results have been obtained in a study of the effect of the mercurial salyrganic acid upon oxyhemerythrin.

Hemerythrin from *Phascolosoma gouldii*, crystallized by dialysis in the cold against 20% ethanol, was dissolved in borate buffer of pH 8.1 and ionic strength 0.08. Addition of salyrganic acid (Hg/Fe ratio approximately ½) caused a rapid loss of oxygen by the pigment as judged by the disappearance of color. Oxyhemerythrin shows absorption maxima, in addition to the protein band at 280 mμ, at 330 mμ and 500 mμ. In the presence of mercurial an additional peak appears at 375 mμ, whereas that at 500 mμ disappears. Either of these latter peaks may be used to follow the effect of the mercurial. At room temperature the reaction took about one-half hour.

The primary affinity of the mercurial is for sulfide groups. It seems, therefore, that the iron in hemerythrin must be bound to the protein at least in part through a sulfide linkage, and that the scission of the S-Fe bond results in the release of bound oxygen. Since there are approximately three Fe atoms per mole of O_2 in fully oxygenated hemerythrin, it was of interest to determine quantitatively the minimum amount of mercurial required to deoxygenate the protein. Titration experiments with salyrganic acid were carried out at approximately 3° C. to minimize the spontaneous conversion of hemerythrin to methemerythrin. The results indicate that one mole of mercurial per three iron atoms completely deoxygenates hemerythrin. Thus there seems to be only one sulfide group per three iron atoms and it appears possible that only one of the iron atoms is actually linked to O_2.

Hexokinase and isomerase activity in eggs of the sea urchin, Arbacia punctulata, and other marine forms. M. E. Krahl, A. K. Keltch, C. P. Walters and G. H. A. Clowes.

The following values for hexokinase activity in homogenates of eggs and embryos were obtained (micrograms glucose used at 20° C. per hour per mg. protein); unfertilized eggs, 67; eggs fertilized one hour, 72; 24-hour plutei, 155; 48-hour plutei, 226. The hexokinase activity of the unfertilized egg homogenates was virtually all recovered in the supernatant fraction when the homogenate was centrifuged at 20,000 g. for 30 minutes. Relative rates of phosphorylation of various sugars by the supernatant fraction when saturated with substrate were, approximately: glucose, 1.0; mannose, 1.2; fructose, 1.8; 2-desoxyglucose, 2.0; glucosamine, 0.6; galactose, 0.0; fructose-6-phosphate, 0.2; glucose-6-phosphate, 0.0. The rate in presence of saturating concentrations of two substrates was in no instance significantly greater than the highest rate for one substrate of the pair; thus, there is no evidence for more than one hexokinase. The concentrations for half maximal hexokinase activity with various substrates were, approximately: glucose, 0.00003 *M*; fructose, 0.00075; mannose, 0.00007; 2-desoxyglucose, 0.00025. The *Arbacia* hexokinase differs from that of other animal tissues with respect to the concentrations of various sugars required for saturation, as well as in the relative maximum rates. Hexokinase activity was found in eggs of another echinoderm, *Echinarachnius*, but none in eggs of the annelid, *Chaetopterus*, or of the mollusc, *Mactra*. The isomerase activity in the supernate fraction from *Arbacia* is sufficient to convert glucose-6-phosphate to fructose-6-phosphate at about 20 times the rate at which the hexokinase present can form glucose-6-phosphate from glucose. No myokinase was present in the supernatant fraction.

Cholinesterase and ciliary activity in the gill of Mytilus. Samuel P. Maroney, Jr., and R. R. Ronkin.[1]

Recently Seaman (1951) and Kordik (1952) have indicated a relationship between cholinesterase and ciliary activity. In the present investigation, cholinesterase activity of ctenidial (= gill) homogenates of *Mytilus edulis* L. was determined by the standard Warburg procedure for carbon dioxide production. Although the rate of spontaneous breakdown of acetylcholine was not large enough to be detected under our experimental conditions, the rate of hydrolysis in the presence of homogenate increased to $Q_{ACh} = 3.47 \pm 0.20\lambda$ CO_2 per hour per mg. (dry). The enzymic activity was completely inhibited by eserine at concentrations of 10^{-5} *M* or greater.

The rate of movement of gill fragments along the bottom of a Petri dish of buffered (0.03 *M* glycine, $pH = 8.0$) sea water was used as a measure of the rate of ciliary movement. The effect of eserine on ciliary activity was tested by measuring the rate of movement before and after the addition of the inhibitor. The effect of eserine was two-fold: ciliary activity was reversibly inhibited at 10^{-3} *M* eserine and was reversibly enhanced at 10^{-4} *M* and 10^{-5} *M* eserine. The concentration of eserine for maximal enhancement of ciliary activity, 10^{-4} *M*, did not have a measurable effect on oxygen consumption (Warburg) of gill fragments.

If one assumes that the inhibitor is effective in the interior of the intact cell at the same concentration as that which obtains outside, the complete inhibition of cholinesterase activity plus the apparent failure to inhibit oxygen consumption of fragments with enhanced ciliary movement, suggest that there may be a relationship between this enzyme and ciliary activity in *Mytilus.*

The electrochemical potential difference of the chloride ion in the giant squid axon-sea water system. Alexander Mauro.

It has been postulated many times in the literature on axon physiology that the chloride ion, among others, might be in thermodynamic equilibrium with the extracellular fluid. This condition can be determined unambiguously by invoking the electrometric property of a pair of identical reversible electrodes, namely, that the electrostatic potential developed at the metallic wires of the electrode system is proportional to the electrochemical potential difference existing between the electrode coordinates. Thus if a condition of thermodynamic equilibrium were to exist between two phases the electrostatic potential developed by the electrode pair should be zero.

An attempt was made to construct a reversible silver-silver chloride electrode by cementing a fine silver wire (15 micra) in a glass capillary. The ability of the electrode to behave as a "chloride electrode" was investigated by observing the potential developed by the electrode versus a calomel half-cell in solutions of varying chloride concentrations. Moreover, the electrode maintained a stable "zero" potential of fractions of a millivolt for many days versus a macroelectrode in a solution of given chloride concentration.

Although this communication is to be regarded as a mere preliminary note, measurements on twelve axons in sea water seem to indicate that the chloride ion is not in thermodynamic equilibrium. This conclusion is warranted by the fact that the "resting potential" as developed between the internal and external silver-silver chloride electrodes was of the order of thirty-five millivolts (internal negative).

Effect of some antibiotics on the cleavage of Arbacia eggs. Yoshiaki Miura.

During the course of an investigation to find new antibiotics which will be effective in curing ascites tumor of rats, Umezawa *et al.* recently have found two effective substances from *Streptomyces thioluteus,* designated as mitomycin and 4-A-1. The present investigation was undertaken to test the effects of these and other antibiotics on the cleavage of Arbacia eggs.

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[1] Supported by the Faculty Research Committee of the University of Delaware, and by a contract between the University and the Office of Naval Research, Department of the Navy (NR 160–015).

The concentration of test substances which inhibit or retard cleavage of Arbacia eggs are, respectively: Aureothricin (*Streptomyces albus*), 10 mg./L and 1 mg./L; 4-A-1, 100 mg./L and 10 mg./L; Achromycin (*Streptomyces alboniger*), 1000 mg./L and 100 mg./L; Mitomycin, 100 mg./L and 10 mg./L; colchicine, 500 mg./L and 20 mg./L; podophyllotoxin, 10 mg./L and 5 mg./L; amino-chlor dibenzofurane (a synthetic usnic acid derivative), 10 mg./L and 1 mg./L. Saturated solutions of Actinomycin-A (*Actinomyces flavus*) and Viscosin (*Pseudomonas viscosa*), even at 100 mg./L, only slightly retarded the cleavage rate. Aureomycin shows complete and irreversible inhibition at 1000 mg./L but no inhibition or even retardation at 100 mg./L. Amino-chlor benzofurane showed irreversible inhibition at 10 mg./L. The actions of the other antibiotics except mitomycin are reversible when the eggs are removed to fresh sea water after one hour immersion with inhibitory solution.

Mitomycin displays a distinctive kind of action on the eggs. The total inhibition of the cleavage occurred at the concentration of 100 mg./L. At 10 mg./L, the first cleavage was retarded but morphologically normal until near the end of the furrowing process. Then cytoplasmic blisters appeared in a contrast group on both blastomeres, at the outer edge of the furrow. As a rule these eggs did not divide again, although eggs which did not bleb underwent a second cleavage. These images are entirely different from those which one can observe with colchicine or podophyllotoxin.

Qualitative analysis of acid-soluble, purine and pyrimidine bases in developing Echinarachnius eggs. YOSHIAKI MIURA AND ROBERT J. NEFF.

Aliquots (containing 3–4 × 10^5 individuals) of *Echinarachnius* unfertilized eggs, or first cleavage, early blastula, early gastrula, and prism embryos were examined. Samples were packed centrifugally, chilled in a cracked-ice bath, homogenized in 1.5 ml. of cold 1% perchloric acid, and cold-extracted twenty minutes. Residues were removed by centrifuging 10 minutes at 10,000 rpm in a Serval high speed centrifuge and the supernatants used for analysis by chromatography on Whatman number one paper at room temperature. The three following solvent mixtures were used in the chromatography: (1) n-butanol, 3 vols.; ethanol, 2 vols.; 5 N HCl, 2 vols.; (2) n-butanol, 86 vols.; water, 14 vols.; (3) solvent (2), 95 vols.; 28% NH_4OH, 5 vols. Synthetic purine and pyrimidine bases were used as controls in the chromatograms. Spots of known and unknown samples were detected with an ultraviolet lamp and Rf values calculated. Spots were eluted with 0.1 N HCl and further characterized by their ultraviolet absorption spectra. The small amount of echinochrome pigments in *Echinarachinus* eggs did not interfere with the analyses.

Appreciable amounts of guanine and adenine were found in all stages examined. Hypoxanthine was present up through the gastrula stage, but was not detectable in the prism stage. Xanthine appeared only in the prism stage. There was also an unidentified base having the same Rf value as uracil present in unfertilized eggs and embryos up through the gastrula stage. Like hypoxanthine, this material disappeared after gastrulation. That this material was not uracil was indicated by its absorption maximum and minimum in 0.1 N HCl. They were 268 and 245 millimicrons, respectively, for the unknown material, as compared to 260 and 230 millimicrons for uracil extracted and read under the same conditions.

Protamin in an extract of the sperm of Nereis limbata. W. J. V. OSTERHOUT.

Nereis limbata was collected from the Marine Biological Laboratory wharf on the Eel Pond before June fifteenth. Soon after collecting, the "dry sperm" was obtained as a soft white mass, avoiding contamination as much as possible. A standard solution of salmon protamin was used to determine the adequacy of the method employed. The suspension of live sperm in sea water at various concentrations showed absence of protamin when the suspension was centrifuged and the supernatant liquid tested.

When sufficient sperm was suspended in sea water and heated at 55 degrees centigrade for ten minutes, allowed to stand for several hours after which it was centrifuged and the supernatant liquid was removed and tested, the presence of protamin was clearly indicated. In the absence of the reagent the test was negative. This indicated that protamin was extracted from the dead sperm by heating. An extract of the dead sperm capable of activating the egg was too dilute to show a test for protamin. A similar result was obtained with *Nereis limbata* collected from Vineyard Sound in August except that the season was too late for the activation experiment. Salmon protamin in sea water at a concentration that gave a positive protamin test produced no persistent foam when bubbles of air were passed through it before and after heating for ten minutes at 55 degrees centigrade. This indicated that the salmon protamin was not surface active.

Surface active material obtained from Nereis limbata. W. J. V. OSTERHOUT.

Nereis limbata was collected from Vineyard Sound. Soon after collecting, the "dry sperm" was obtained as a soft white mass, avoiding contamination as much as possible. The sample consisted of one part of "dry sperm" and nine parts of filtered sea water. This was centrifuged. The supernatant liquid was removed and bubbles of air passed through it at room temperature. Persistent foam was produced which indicated the presence of surface active material. The same test for surface active material was made in subsequent experiments. The supernatant liquid was replaced by an equal volume of sea water which was mixed with the sperm and centrifuged. This supernatant liquid was removed and the test showed little or no surface active material. This indicated that it had been removed by one washing. On heating this supernatant liquid for ten minutes at 55 degrees centigrade and testing, no surface active material was detected. This indicated that no surface active material was produced by heating. The supernatant liquid was replaced by an equal volume of sea water which was mixed with sperm. This was heated for ten minutes at 55 degrees centigrade and centrifuged. The supernatant liquid was removed and tested. This showed the presence of surface active material. This indicated that surface active material was extracted from the dead sperm on heating. A similar result was obtained when the "dry sperm," appearing as a soft pink mass, was contaminated with blood and possibly other material from the body.

The pH values of the supernatant liquids above described were a few tenths of a pH lower than that of the sea water but on heating them the pH values became about the same as that of the sea water indicating the presence of volatile material, possibly carbon dioxide. These differences in pH values did not affect the surface activity.

Release of an anticoagulant from irradiated Spisula eggs.[1] PETER RIESER AND ALVIN M. KAYE.

In the hope of obtaining information concerning the effect of roentgen rays on the colloidal properties of protoplasm and in the hope also of interpreting the heparinemia occurring in the blood of mammals as a result of whole body irradiation, an attempt was made to demonstrate the release of heparin or heparin-like substances from isolated irradiated cells. The eggs of the clam Spisula contain a powerful anticoagulant (Thomas, 1951). These eggs were suspended in sea water and washed five times to remove the jelly surrounding them. One-half of the suspension was then exposed to 200,000 r, the other half used as a control. Both lots of eggs were then centrifuged, and the supernate incubated with crystalline trypsin, which was subsequently destroyed by boiling. The resultant solutions were centrifuged and the supernate assayed for anticoagulant activity with sheep plasma, using purified thrombin as an activator. The solution from irradiated eggs produced delays of clotting of as much as 48 hours, whereas the control solution from non-irradiated eggs allowed clotting to occur within a few minutes. Addition of toluidine blue to the solutions from the irradiated eggs produced strongly metachromatic precipitates, whereas similar additions to the control solutions did not. Removal of this precipitate from the solutions destroyed their anticoagulant activity. Preliminary studies on the eggs of the sea urchin Arbacia and the worms Chaetopterus and Arenicola showed no effect of radiation on the release of anticoagulants. This may in part be due to the fact that Spisula eggs produce more anticoagulant; in part also to the fact that the Spisula anticoagulant has a more powerful effect on vertebrate blood (*cf.* Thomas, 1951). Similar investigations are planned for various types of vertebrate cells.

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[1] This investigation was supported by a research grant from the U. S. Atomic Energy Commission, administered by L. V. Heilbrunn.

Surface activity of hypobranchial mucus of Busycon: possible nutritive function of mucous substances. R. R. Ronkin.[1]

Fresh hypobranchial mucus of the large prosobranch gastropod, *Busycon canaliculatum* (L.), lowers the interfacial tension between air and the sea water in which it is dissolved. The lowest value obtained, 47.5 dynes $cm.^{-1}$, was 64% of that obtained with pure sea water (du Noüy method; $t = 22°$ to 25° C.). Graphical solutions of the Gibbs adsorption equation indicate that the greatest excess of solute concentrated in the surface phase occurred when the mucus was diluted about 10 times with sea water. Estimates of solute concentration suggest that under these conditions the concentration (w/v) of mucus in the sea water was between 0.01% and 0.05%. Although it is certain that different animals produce mucus with widely diverse behavior in solution, it is possible that the surface activity of some types of mucus solutions is important in feeding, in that it improves the wettability of food particles.

The effect of x-rays on the ability of marine egg protoplasm to undergo two colloidal reactions. Alburt M. Rosenberg.[2]

X-irradiation may not produce a measurable colloidal change in the unfertilized egg but may alter the ability to undergo such required changes (Wilson, 1950). In two situations involving colloidal change, the behavior of irradiated eggs was compared with that of control eggs.

Cerebratulus lacteus eggs in the metaphase of the first maturation division were treated with sea water made hypertonic with calcium chloride (Yatsu, 1905) to form numerous asters in the cytoplasm. The cytasters were observed in fixed whole-mount preparations under phase microscope and in the living cells with dark field illumination. Data gathered a half hour after final treatment showed eggs irradiated with 100,000 r, a dose which significantly reduced the size of the metaphase figure, have many very small vesicles (cytasters in the fixed) while the non-irradiated had fewer but larger vesicles. This difference is caused by the many vesicles originally present in the control coming together as they approach the center of the cell. The viscosity of the eggs with cytasters was considerably higher than that of untreated eggs.

Arbacia punctulata eggs put into hypotonic sea water usually break at one point. Several seconds later these eggs appear suddenly pink under dark field. The time from breaking at the cell membrane to pigment granule lysis was slightly longer for eggs previously given 20,000 r than for the control. However, experimental reproducibility was not entirely satisfactory.

Future research should include determinations of viscosity in irradiated and control eggs subsequent to treatment with agents which increase and which decrease the viscosity of the protoplasm. Perhaps the action of x-rays may best be understood not as an alteration of the colloid itself but rather as an effect on the agents which bring about colloidal changes in the living cell.

Persistent diurnal rhythms of O_2-consumption in the periwinkle, Littorina littorea, and the oyster drill, Urosalpinx cinereus. Muriel I. Sandeen, Grover C. Stephens and Frank A. Brown, Jr.

The O_2-consumption of the common periwinkle, *Littorina littorea,* was studied using an automatic, continuously-recording respirometer. A group of 6 or 8 snails was placed in each of four respirometers and their respiratory rate followed for about a 48-hour period. These were then replaced by freshly collected animals. Throughout a period of 26 days a total of about 400 snails was used. It was found that the average value of O_2-consumption in all experiments was about 9 ml./kg./hr. Large variations in rate of O_2-consumption were observed in each daily period. These variations in oxygen consumed occurred in such a manner as to suggest the existence of a diurnal pattern. For two 15-day periods the average O_2-consumption per snail per hour was tabulated and an average value obtained for each hour of a daily period. In this way any possible persistent tidal variations were eliminated. The two resulting daily curves of O_2-consumption clearly demonstrated the existence of a diurnal rhythm in metabolism. The metabolic rate is lowest about 2 to 3 A.M., increases rapidly to a maximum value about 8 to 9 A.M., and declines steadily to a second minimum value about 2 P.M. Thereafter, there is a gradual increase to a second maximum at approximately 10 P.M. The values through the daily cycle ranged in magnitude from a minimum of about 5 to a maximum of about 13 ml./kg./hr. A comparable investigation of the snail Urosalpinx revealed a similar persistent diurnal rhythm in metabolism but with times of maxima and minima slightly different.

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[1] Aided by a grant from the Faculty Research Committee of the University of Delaware, and by a contract between the University and the Office of Naval Research, Department of the Navy (NR 160–015).

[2] Predoctoral Fellow, Atomic Energy Commission.

Osmotic and other studies of the Spisula egg. VICTOR SCHECHTER.

The osmotic and tensile properties of the Spisula egg were studied by the means of allowing the cells to come into equilibrium with diluted sea water of graded concentrations. Preliminary tabulation of results indicates a linear relationship between dilution of the medium and the amount of swelling of the egg, in the range where no damage to the egg surface occurs. Data were also obtained on the osmotic properties of the nucleus, which enlarges measurably when the egg is placed in a hypotonic medium. Characteristic indentation of the cortex, which is a feature of aging of the unfertilized Spisula egg cell, can be induced with vitamin K. This effect does not occur in the absence of calcium. The effect of vitamin K upon the osmotic properties of the egg membrane is under investigation.

Discontinuous carbon dioxide output by diapausing pupae of the giant silkworm, Platysamia cecropia. HOWARD A. SCHNEIDERMAN AND CARROLL M. WILLIAMS.

Using the "direct" Warburg manometric method, it has been shown that diapausing pupae of the Cecropia silkworm release carbon dioxide in discrete bursts, confirming the diaferometric CO_2 measurements of Punt on other species. Oxygen consumption, by contrast, exhibits no such discontinuities. In a typical pupa weighing five grams and having a steady oxygen uptake of 70 $mm.^3$/hour at 25° C., the sudden release of CO_2 occurred regularly once every eight hours. At lower temperatures the bursts were less frequent; at 10° C. they occurred about once every three days. Each burst lasted about 30 minutes and accounted for 95% of the total CO_2 output. The remaining 5% was given off continuously. The over-all R. Q. measured over a two-day period was 0.78. That virtually all the gas exchange takes place via the spiracles may be shown by occluding them with wax.

The frequency of CO_2 bursts increases with increasing metabolic rate until, when oxygen uptake exceeds 160 $mm.^3$/gram live weight/hour, the bursts disappear and CO_2 evolution becomes continuous. Such animals when placed at 10° C. show a reduction in metabolism, and CO_2 output again becomes discontinuous.

A larger percentage of the total CO_2 was given off continuously at high burst frequencies than at low burst frequencies; when bursts occurred at one-hour intervals, more than half the CO_2 was released continuously in the inter-burst period.

Exposure to 6% O_2, while not affecting O_2 uptake, resulted in the disappearance of CO_2 bursts. Exposure to 10% CO_2 also eliminated the discontinuous release of CO_2. Isolated anterior halves of pupae and isolated pupal abdomens both gave off CO_2 bursts.

An explanation of the burst phenomenon consistent with the above observations will be offered subsequently.

A rare poly-anomalous embryo. D. R. SHANKLIN.

In with several hundred normal *Fundulus heteroclitus* embryos, a four-day specimen was found anomalous. It was followed to eight days, dechorionated, fixed in Bouin's, serially sectioned, and stained with BPB. These external anomalies were apparent: monophthalmia with two lenses; no oral depression; a bulbous mass of red tissue in place of the right pectoral fin;

similar tissue adjacent to the tail fin; a single hypertrophied urinary vesicle, without its midline constricture. Proceeding caudad, an inspection of the sections shows the following anomalies: reduced telencephalonic evaginations, which are displaced dorsocaudad to the monophthalmia, which opens ventrally; diencephalonic fusion with reduction of the third ventricle to a cord of ependymal cells; absent oral cavity; the lining cells of the pharynx contain a dense granular metachromatic material which may be the hatching enzyme precursor; the mesencephalonic aqueduct is typical for a brief spell, then vanishes; the fourth ventricle is enlarged, and the alar plates are absent; a discrete cleft occurs between ventral basal plate and notochordal tissue, concurrent with the auditory vesicle, which is enlarged; the pectoral fin dismorph, an aberrant splenic primordium, and the greatest accumulation of the metachromatic material occur at the decresence of the myelencephalon; median pronephri are joined by lateral pronephri, which proceed through many sections and fuse caudad to the convolutions, the left pronephros crossing the midline and continuing thus to the vesicle; the swim bladder is displaced caudad and to the right; here the sclerotomic mesoderm is replaced by transversely oriented striate muscle, the band intermittently passing to fuse with the longitudinal bundles about the spinal column; the aberrant tail tissue is also splenic.

The development of isolated blastomeres of Fundulus heteroclitus.[1] John R. Shaver.

Previous analyses of the developmental potencies of the early blastomeres of *Fundulus* have been made by destroying cells and observing the development of surviving blastomeres. The present experiments parallel those of Tung on *Carassius,* in that both fragments resulting from the separation of the 2- or 4-cell stages have been raised. After dechorionation in sterile medium, the blastoderm was separated along the first cleavage furrow continuing through the yolk-sac. From one to two thirds of the yolk was lost in the operation, and from over 200 such isolations only 50 pairs of fragments successfully healed and cleaved. Cleavage followed an essentially normal pattern in most cases, and a majority of fragments proceeded to gastrulate, either partially or completely, apparently depending on the amount of yolk proportional to the blastoderm. Controls consisted of 2-cell stages dechorionated but otherwise unoperated. A very small percentage of the healed isolates proceeded beyond gastrulation and in no case so far have embryos been obtained from both members of the pair or fragments. One case of a normal but small embryo has been gotten from one of the first two cells; the other member of this pair did not form an embryonic axis. Another small but normal embryo developed from two of the first four cells. In many cases embryonation proceeded in one or both fragments to some extent but failed. Much reduced embryos, poorly differentiated, resulted in about 10% of cases. Most numerous were fragments in which vesicular forms, with or without "tail-like" structures protruding from the blastoderm, developed; in some of these blood and/or pigment cells differentiated on the yolk-sac.

Surface active properties of an extract of the sperm of Nereis limbata. Theodore Shedlovsky and W. J. V. Osterhout.

It has been stated by one of us (this journal, Vol. 103, No. 2, 305–306, October, 1952) that an extract of dead sperm which can activate the egg contains surface active material, and that purified Duponol in sea water also was found to be activating.

Extract, S, of dead sperm in sea water did not alter the osmotic pressure of sea water within one per cent at the concentration capable of activating the egg.

The surface tension of various dilutions of S showed a rapid decline from zero concentration reaching an essentially constant value of about 2/3 the surface tension of sea water above ten-fold dilution of S. A similar surface tension-concentration curve was obtained with purified Duponol which reached a surface tension of about 1/2 that of sea water above 0.002% concentration. This corresponds to the critical concentration above which micelles are formed. Studies of surface films revealed that S forms high surface viscosity films, with slow drainage up to 55° C. above which no stable films could be made. In contrast, purified Duponol films drained rapidly even at 0° C., showing relatively low surface viscosity. Such behavior is characteristic of foam-producing substances free of slightly soluble alcohols or fatty acids. When such are present slow draining films with high surface viscosities are formed, but these can be transformed into rapidly draining films by raising the temperature. It appears that the surface active properties of S may be due to one or more foam-producing substances which also contain a slightly soluble material such as an acid or alcohol. Since surface tension (liquid-gas) behavior is not necessarily paralleled in interfacial systems it would be of interest to continue these studies with observations on sperm extract-oil interfaces.

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[1] Supported by funds from U. S. Public Health Service.

The role of the vitelline membrane in the fragmentation of the Arbacia egg by centrifugal force. SISTER FRANCIS SOLANO AND DANIEL MAZIA.

The centrifugal force required to break a cell into two fragments has been used in the study of the physical properties of the cell surface. It has been recognized that the measurements apply to a complex of surface layers. In the sea urchin egg, one of these is the vitelline membrane which is transformed into the fertilization membrane on activation. This membrane may be removed by means of proteolytic enzymes, after which activation and development may occur, but no fertilization membrane is seen.

A mixture containing one mg. of crystalline trypsin and one mg. of crystalline chymotrypsin in sea water was effective in removing the vitelline membrane of *Arbacia* eggs. Such eggs fertilized and developed normally. Control eggs and eggs from which the membranes had been digested were centrifuged simultaneously. None of the controls fragmented at the limiting force at which 100% of the membraneless eggs fragmented. A force at least 25% greater was required for fragmentation of the controls. Therefore, the vitelline membrane contributes significantly to the resistance of the egg to fragmentation, and is an important component in what has been described as the tension at the surface of the egg.

It had been found earlier by E. S. G. Barron that uranium ions in low concentration greatly increase the resistance of eggs to fragmentation. We have confirmed this, employing 5×10^{-4} M uranyl nitrate, and obtain the same results in the presence and absence of the vitelline membrane.

Electric current distribution in a multicellular system as determined by the pattern of biological events resulting from exposure of developing Rana pipiens eggs to externally applied elliptically polarized currents. RICHARD N. STEARNS.

Developing *Rana pipiens* eggs were exposed to elliptically polarized electric currents, the frequency of rotation of the current vector varying between zero and 6000 cycles per second. This type of current pattern was used to eliminate membrane polarization effects. The eggs were in the field for ten minutes at different stages of development, and visible damage was assayed immediately under a dissecting microscope. Changes in developmental pattern were followed in whole embryos and in histological sections. The distribution of events arising when eggs are treated between 0.25 and 5.0 times threshold at different developmental stages are as follows: 1) The damage is mainly restricted to the interfaces between cells. 2) This damage pattern does not result from a corresponding sensitivity pattern since the per cent of eggs which show visible damage restricted solely to the interfaces at the 2-cell, 4-cell and 8-cell stages, are 10%, 50%, and 100%, respectively, with the voltage range employed in these experiments. 3) The per cent mortality decreases when eggs are treated with greater cell numbers. 4) The per cent of eggs which cleave but do not differentiate is low when treated before first cleavage, rises sharply at the 2-cell stage and declines with a slope approximately like that of the mortality curve. 5) The per cent of eggs where only the vegetal half is destroyed is similarly related to cell number. (6) Alteration in developmental pattern is low before first cleavage, rises sharply at the 2-cell stage, declines with increased cleavage number, and rises again at approximately mid-cleavage and again decreases between small yolk-plug and tail-bud stages. These results indicate that the biologically effective current is an extracellular current (an ionic current as indicated by the threshold-frequency curves) such that cell membranes must be damaged before the interior of the egg is affected, when the cell number is greater than one.

Alteration in developmental pattern arising from cell membrane damage caused by elliptically polarized electric currents in Rana pipiens eggs. RICHARD N. STEARNS.

The eggs described in this abstract were ones treated in the manner described in the previous abstract. Neurulae which had prior electrical treatment stop visible differentiation at the tail-bud stages for 24–56 hours at 18 degrees after which time 85% of the stopped embryos become normal tadpoles. The irreversibly inhibited embryos show non-specific damage in the three germ layer derivatives, or differentiation may be stopped entirely. If the voltage gradient range is increased to 6 times threshold, the per cent of irreversibly inhibited embryos increases to 50%.

Embryos may be stopped in gastrulation by treatment as early as the 2-cell stage and as late as the small yolk plug-gastrulae stages. After a period where no obvious mass cell movements are observed, development proceeds without gastrulation, the embryos oriented with reversed dorso-ventral axes. The embryos are internally twinned, the two systems forming directly across the yolk-plug and a swimming tadpole will result with a regionally well-differentiated nervous system. Thus the regional differentiation has occurred without gastrulation.

Animal caps, which are produced by destroying the vegetal halves of developing eggs and subsequently left in contact with the killed material, will differentiate ectodermal, mesodermal, and endodermal derivatives, development proceeding in the same way as the stopped gastrulae. In eight cases, swimming tadpoles resulted with good regional differentiation. Two caps which healed over so that they did not remain in contact with the killed area showed no differentiation. Thus it is suggested that the gross geometry is important not only for histogenesis but also organogenesis, and the killed material may also play a purely chemical role in the differentiation and organization of the animal cap.

Membrane potential changes in fibers of the frog sartorius muscle during sodium extrusion and potassium accumulation. WILLIAM K. STEPHENSON.

When nerve or muscle is in a steady-state condition, the membrane potential may be expressed by the familiar Nernst equation. In accord with this expression, when K^+ is added to the bathing solution the potential decreases; however, if K^+ is injected into the fiber (Grundfest and others), the membrane potential does not increase in accord with theory. This report deals with membrane potential changes taking place in muscle fibers greatly depleted of K^+ during recovery from this condition.

Frog sartorii were dissected and soaked for 24 hours in K-free saline ($0.12\ M$ NaCl + $0.005\ M$ $Na–PO_4$ buffer, pH = 7.2) at 2° to 6° C., during which time muscle K^+ content fell to 30.3 ± 4.6[1] mM./Kg. fiber weight (mM.) and Na^+ increased to 98.2 ± 5.3 mM. These muscles were then transferred to high-K recovery saline ($0.11\ M$ NaCl + $0.01\ M$ KCl + buffer) at room temperature for 55 minutes, where their K^+ content rose to 72.2 ± 3.1 mM. and Na^+ dropped to 59.2 ± 5.4 mM. Membrane potentials were measured at the beginning and end of the recovery process by the Ling and Gerard glass microelectrode technique, and readings of 43.9 ± 1.6 mV. and 46.1 ± 1.5 mV., respectively, were obtained. Control muscles suffered drops of membrane potential during recovery as follows: soaked and recovered in K-free saline, 46.3 ± 1.9 mV. to 41.3 ± 1.9 mV.; and soaked and recovered in high-K saline, 52.3 ± 0.9 mV. to 48.1 ± 0.9 mV.

Calculations of the membrane potentials expected on the basis of the Nernst equation give values of 28 mV. before recovery and 50 mV. after recovery, yet the recorded potential at the onset of recovery was markedly higher than theoretical and that at the termination, somewhat lower. These results indicate that the membrane potential is not necessarily dependent upon the K^+ distribution, and that the Nernst equation can not, therefore, be applied in cases of rapid ion shifts.

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[1] Standard Error of the mean.

(This work was aided in part by Contract Nonr–24900, Office of Naval Research, administrated by H. B. Steinbach.)

A note on specific pigment in the lateral line receptor cell. GEORGE C. THRASHER.[1]

Recently Allison (1953) has suggested that sight, smell and hearing all require the presence of pigment for the proper performance of their receptor cells. This investigation is concerned with the pigment of still another group of receptors, those of the lateral line system. Bedell (1939) called attention to this pigment in the hair cells of lateral line organs of tadpoles and salamander larvae. In freshly collected specimens it appears as clumps of bright orange-yellow granules. These survive sectioning and routine staining. Two questions immediately arise about this material: (1) Is it necessary for the proper performance of the receptor cells? (2) What is its chemical nature? If the answer to the first question is yes, then it would seem that an identical or at least very similar pigment would be required by other forms possessing a lateral line system, particularly fishes. Whole and teased fresh mounts and stained and unstained frozen sections of lateral line organs of dogfish, skate, catfish and others were examined for granules resembling those apparent in similarly treated organs of tadpoles and salamanders. None were observed. If this finding is substantiated by further investigation it would seem to indicate that the pigment of these receptors is of no functional significance as far as the reception of impulses or the transmission of stimuli is concerned. The initial color of the known granules and the color changes which they undergo as they degenerate under laboratory conditions are reminiscent of rhodopsin (visual purple) changes. Histochemical tests, however, demonstrate that the granules are not carotene or carotenoid.

Nucleic acid turnover rates in Arbacia eggs and sperm. CLAUDE VILLEE, DOROTHY VILLEE AND RUTH LAPLACE.

A solution containing 25 microcuries of P^{32} in 0.2 ml. of sea water was injected into the coelomic cavity of adult *Arbacia*. The urchins were then kept in running sea water for 24 hours, after which eggs and sperm were collected either by KCl injection or by dissection. The eggs and sperm were washed in sea water, and then an aliquot of the eggs was fertilized by sperm from uninjected males and allowed to develop for eight hours. The sperm, unfertilized eggs and fertilized eggs (swimming gastrulae) were killed by the addition of trichloracetic acid, homogenized, and fractionated by the method of Schmidt and Thannhauser. Phosphorus was determined by the method of Fiske and Subbarow and radioactivity by a Geiger dip counter. The experiments showed that no P^{32} was incorporated into the desoxyribonucleic acid (DNA) of the sperm *in vivo*, but some was incorporated into the sperm ribonucleic acid (RNA) fraction. Radiophosphorus was incorporated into the RNA of unfertilized eggs *in vivo* slightly faster than into the DNA. Upon fertilization and during development in P^{32}-free sea water, the amount and specific activity of the DNA fraction increased two- to four-fold, whereas the amount and specific activity of the RNA fraction increased only slightly. The DNA of mature sperm, in contrast to that of unfertilized eggs, does not incorporate phosphorus *in vivo*. A comparison of the specific activities suggests that the phosphorus of the DNA synthesized during early development is derived from the acid-soluble phosphorus fraction.

Relationship between time of day and inhibiting influence of low temperature on the diurnal chromatophore rhythm of Uca. H. MARGUERITE WEBB, MIRIAM F. BENNETT, ROBERT C. GRAVES AND GROVER C. STEPHENS.

Exposure of *Uca pugnax* for short periods to a temperature of 5° C. was found to inhibit the diurnal rhythm as observed in the black chromatophores. In one experiment groups of

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[1] This work was supported by a Massengill Fellowship.

50 animals were placed in a darkroom at 7 P.M. and remained in the dark for the duration of the experiment. Each group was exposed to a temperature of 5° C. (in a refrigerator) in darkness for a single 6-hour period in a different quarter of the day. Following this treatment the average stage of the chromatophores was determined at hourly intervals from noon until the animals reached the night phase on five successive days. In all cases a shift in the phases of the diurnal rhythm was observed but in no case did the extent of the shift equal the chilling period. When the extent of inhibition was calculated by comparison with a control group, it was seen to vary in a regular manner with the time of day at which the animals were chilled. The times of day and amounts of inhibition obtained were as follows: 12 P.M. to 6 A.M., 72%; 6 A.M. to 12 noon, 56%; 12 noon to 6 P.M., 63%; and 6 P.M. to 12 P.M., 78%. When two-hour chilling periods were used, 79% inhibition was found when chilling occurred from 8 P.M. to 10 P.M., and only 24% with chilling from 10 A.M. to 12 noon.

Studies of the effects of x-radiation upon Paramecium: variations among and within species.[1] RALPH WICHTERMAN.

Clones of seven species of *Paramecium* were irradiated in gradually increased dosages up to 500 kiloroentgen using Nylon syringes as irradiation chambers. Specimens were cultivated in lettuce medium with *Aerobacter aerogenes* as the bacterial food and irradiated at the growth phase. Four syringes, free of air, were irradiated simultaneously. Each experiment involved 800 specimens (200 per syringe in 2 cc. culture fluid). After each x-ray dosage, countable numbers—commonly 10—were expressed from the syringes, placed in spot plates in moist chambers and observations continued periodically to 48 hours. Variations in x-ray susceptibility exist not only among species but within them. Under fairly uniform cultural and x-ray conditions, if percentage of survivors is computed against x-ray dosage and attempts made to obtain an LD 50 for 24-hour periods, it is observed that the species may be arranged in sensitivity to x-rays with *P. calkinsi* the most resistant (LD 50: 400 kr.) and *P. trichium* the most sensitive (LD 50: 170 kr.) as follows: *P. calkinsi, P. multimicronucleatum, P. bursaria, P. caudatum, P. aurelia, P. polycaryum, P. trichium.*

X-ray susceptibility is dependent not only upon the concentration of bacteria and dissolved oxygen in the medium with paramecia, the chemical constitution of the culture fluid and changes brought upon it as a result of ionizing radiations (such as production of hydrogen peroxide) but also the phases and stages of growth of individual paramecia. When the results of many experiments are plotted to yield per cent survival curves they frequently are not sigmoid. Instead the slope of the curve is so steep approaching lethality as to be almost vertical. However before the steep drop, commonly two peaks occur. These peaks or variations from the typical curve are greater than the biological variation expected and indicate definite sensitivity levels and differences in specimens being irradiated. These sensitivity thresholds, plus conditions of irradiation and the fact that many immobilized specimens may remain seemingly dead for hours thru the 24-hour period only to recover later from irradiation effects, may explain the difficulty in obtaining consistently uniform LD 50 values.

Pharmacological studies on marine invertebrates. CLARE M. WILBER AND SR. ELIZABETH SETON.

This is a report of progress on a study of the effects of octamethyl pyrophosphoramide (OMPA), hexamethonium (C6), and decamethonium (C10). Changes in the glucose content of the body fluids from *Amphitrite ornata* and *Phascolosoma gouldii* and in the blood from *Libinia emarginata* were recorded. In general, treatment with any of these agents, by immersion or by injection, results in a drop in the glucose of the body fluid from the animals studied. For example, in *Phascolosoma* immersed in C6 (one g. per 1200 ml. sea water) the glucose of the body fluid decreased from an average of 19 to 16 mg. per 100 ml.; in the same concentration of C10 the decrease was from 24 to 20 mg. per 100 ml.; after immersion in OMPA the glucose decreased from 14.2 to 8.5 mg. per 100 ml. body fluid. After injection of 5 mg. of C10 into *Libinia* a similar decrease in blood sugar resulted: from about 5 to 1 mg. per 100 ml. Injections of C6 also brought about a decrease in blood sugar in the spider crab. A similar situation obtains in *Amphitrite: e.g.* after C6 injection a decrease in glucose from 29 to 23 mg. per 100 ml. body fluid; after immersion in OMPA like decreases are found. Since C10 imitates acetylcholine (ACh) in its action and OMPA inhibits cholinesterase, one would anticipate that their effects would be similar. On the other hand C6 antagonizes acetylcholine. The excitation of the animals induced by accumulation of acetylcholine (OMPA) or by the action of an ACh-like material (C10) may deplete the glucose in the body fluids faster than it can be replaced from storage tissues. A study of the tissue glycogen of these animals, treated as in the present work, is planned.

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[1] Aided by a grant from the Committee on Research, Temple University and performed under Contract NR 135–233, Office of Naval Research.

The action of cyanide on the protoplasmic colloid.[1] WALTER L. WILSON AND L. V. HEILBRUNN.

As has been long known, dilute solutions of potassium cyanide prevent the division of marine eggs. The effect has generally been attributed to the fact that cyanide inhibits the activity of some of the respiratory enzymes. But the division of a cell is a mechanical process dependent on forces developed in the protoplasmic colloid. Hence it is of interest to know how the colloidal changes that occur during the division of a cell are affected by cyanide. In the egg of the worm Chaetopterus, the mitotic gelation is prevented by solutions of cyanide —that is to say, the protoplasm in the interior of the egg stays fluid instead of clotting to form a mitotic spindle. Similarly, in the egg of the clam *Spisula solidissima,* prevention of cell division by dilute solutions of cyanide is associated with a prevention of mitotic gelation. On the other hand, earlier work on the egg of the sea urchin Arbacia has been confirmed and in this egg instead of the gelation being prevented, the gelation occurs normally but the protoplasm stays in the gel state and does not return to a fluid condition as it does in the normal untreated egg. Why the protoplasm of the Arbacia egg should behave in one way and the protoplasm of the other eggs in quite a different way is not clear. But in any attempt at interpretation, it should be remembered that cyanide not only inhibits oxidizing enzymes but also acts as an accelerator of proteolytic activity; and proteolytic enzymes and clotting enzymes may be one and the same. In any event, it is hoped that further experiments may help to elucidate the relationship between oxidation and clotting phenomena in the protoplasm.

Colloidal changes during aging of the Chaetopterus egg.[1] WALTER L. WILSON AND L. V. HEILBRUNN.

Much has been written about the colloidal changes that presumably might occur in living cells when they age, but there is little definite information as to the changes that actually do occur. The fact that the cortex of a cell is especially rich in calcium and that the calcium content of cells and tissues as a whole seems to increase during senescence would make it seem probable that the cortex would become richer in calcium as cells grow older. In the egg of the worm Chaetopterus, it is possible to determine the rigidity of the cortex by relatively simple centrifuge tests (Wilson, 1951), and this rigidity seems to depend on the amount of calcium that is combined in the cortex. Soon after the egg is shed into sea water, a force of approximately 7000–9000 times gravity is required to move the granules out of the cortex of the cell. With the passage of time, there is a slow but progressive decrease in the force that is required to move the granules. Thus the rigidity of the cortex declines until some 36 hours after the egg is shed, a force of 1000 times gravity or less has the same effect in moving granules out of the cortex as much larger forces did earlier. Indeed the cortex liquefies to such an extent that the eggs tend spontaneously to disintegrate. Whether the liquefaction of the cortex is due to loss of calcium from it is not certain. However, there is an indication that calcium is released from the cortex to the cell interior. For the interior of the aging egg cell becomes filled with what appear to be vacuoles, and such vacuolization may well be due to a calcium-induced surface precipitation reaction. But there is some doubt as to whether the structures observed in the aging egg are really vacuoles, for there is a possibility that they may be nuclei. This can only be determined by cytological studies of sectioned material and such studies have not yet been made.

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[1] This investigation was supported by a research grant from the National Cancer Institute, National Institutes of Health, Public Health Service, administered by L. V. Heilbrunn.

The effect of strong centrifugal force on the development of Chaetopterus eggs.[1] WALTER L. WILSON AND YONYNAH SCHUB.

Years ago, Lillie found that when eggs of the worm Chaetopterus were centrifuged and then fertilized, development proceeded normally. However, in Lillie's experiments the centrifugal forces used were not strong enough to dislodge the granules from the cortex of the egg, where they are present normally in a single layer. It is possible therefore that the organization of the egg depends on the arrangement of these cortical granules. Wilson (1951) showed that a force of approximately 9000 times gravity, acting for one minute, was powerful enough to move the granules from the cortex. In such eggs development does not proceed normally. Whether the effect of strong centrifugation is due to a movement of the granules or to a weakening of the cortical layer is not yet certain. We have found that forces up to 2600 times gravity, acting for one minute, do not affect normal development. At forces as high as 3500 times gravity, acting for one minute, a few of the eggs developed into motile larvae. None does so following centrifugation for a minute at forces 4300 times gravity. These forces are sufficient to dislodge some but not all of the cortical granules.

A globulite structure for acid insulin sulphate?[2] DOROTHY WRINCH.

In a preliminary study of the dry orthorhombic acid insulin sulphate crystal, Low (1950–3) has recorded principal vector projections and two vector sections. A first question regarding the insulin entities in this crystal, entities with M.W. ca. 11,700 and 12 sulphate groups apiece, is the nature of the polymers of amino acid backbone monomers, N-C_α-C, which form their molecular skeletons. To obtain information as to whether these skeletons are uniaxial, biaxial or genuinely three-dimensional in nature, the vector maps are examined in detail in the neighborhood of the origin. We find no indication of some one particular direction, in or perpendicular to which the distribution of short inter-atomic vectors has special significance. It may be concluded that, for this protein, as for horse hemoglobin and for ribonuclease (Wrinch 1952, 1953), the skeletons are genuinely three-dimensional in their structure and the traditional type of structure comprising a bundle of polypeptide chains may be rejected at the outset. Proceeding with the examination of the vector maps further from the origin, indications are seen, in the low density regions in many directions preceding the high density regions in many directions at about 9–11 Å from the origin, that there may be two globulite structures (separate or associated) in each of these insulin entities, in accordance with the suggestions made regarding trigonal insulin (Wrinch 1948, 1952) in the light of Crowfoot's X-ray data (1946).

LALOR FELLOWSHIP REPORTS

Isolation of protein fractions from echinoderm sperms and eggs. D. HAMER.

Specimens of basic nuclear proteins have been prepared from isolated sperm nuclei of Arbacia, Echinarachnius and Asterias for amino acid analysis. Nuclei were isolated in a saline-citrate medium and then extracted directly with 0.2 M HCl. The acid extracts were dialysed and the protein precipitated as the picrate. The picrates were almost completely soluble in acetone and from the solutions the basic proteins were obtained as the sulphate by addition of a few drops of 2 *M* sulphuric acid. (Only in the case of Asterias sperm was there an appreciable amount of insoluble picrate.) Similar basic fractions are obtained by acid extraction of whole nucleo-protein prepared using strong saline solutions. As is the case with mammalian cells, there was an appreciable amount of protein firmly bound to the nucleic acid and so specimens were also prepared for estimations of the amounts of the major components (nucleic acid, basic protein and acid-insoluble protein).

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[1] This investigation was supported by a research grant from the National Cancer Institute, National Institutes of Health, administered by L. V. Heilbrunn.

[2] This work is supported by the ONR under contract with Smith College.

Preparation of nuclear fractions from unfertilized eggs is a more difficult problem due to the large amounts of non-nuclear material present. Some fractions were obtained, however, by direct extraction of the eggs followed by fractionation of the extracts using the picrate technique described above. In one case (Arbacia) it was possible to prepare corresponding fractions from fertilized eggs. Analysis of this protein may give information about the stage at which the protamine-type basic protein develops into the more complicated histone molecule.

When the amino acid analyses are completed it will be interesting to see if species differences are reflected by variations in the composition of the nuclear proteins, since these are believed to be constituents of the chromosomes.

Oxidation of amino acids by marine animals. D. HAMER.

D-amino acid oxidase has been found to be widely distributed in mammals, particularly in the liver and kidney. Recently other workers have demonstrated for the first time the occurence of this oxidase in invertebrates using Octopus and Sepia.

It has been found that the squid, *Loligo pealii,* contains in the liver an enzyme system capable of oxidizing D-amino acids. Homogenates of the liver were made in 0.5 *M* sodium chloride and the oxidation of amino acids was studied at 25° C. and pH 7.4 using the usual constant volume manometric procedures. The enzyme system was inactivated rapidly at 37° and pH 7.4 and particularly at pH 8.4 which is usually used for determinations on mammalian tissues. The amino acids were used as the DL-forms at a final concentration of 0.02 *M*. Rate of amino acid oxidation was measured as the excess oxygen uptake beyond that due to the endogenous respiration and was expressed as μl. oxygen consumed/hour/mg. homogenate nitrogen. Alanine was oxidized most rapidly of the compounds studied, the mean observed rate being 4.65. Oxidation of leucine and methionine was less rapid and that of phenylalanine still lower (2.8, 2.6, and 1.6, respectively).

Under the same conditions the liver of the smooth dogfish, *Mustelus canis*, oxidized alanine about three times as fast as the squid (12.0 μl/hr./mg. N) but no significant uptake was detected using the other amino acids which were oxidized by Loligo. No oxidation of any of these amino acids was found using homogenates of digestive organs of the starfish (*Asterias forbesi*).

Observations with an interference microscope. A. F. HUXLEY.

The range of usefulness of a two-beam interference microscope, designed and built for quantitative work on isolated cells, was explored by making measurements on a variety of specimens, most of which were provided by other investigators in the Laboratory. The microscope has a water-immersion objective (n.a. 0.9), and the retardation of light by the specimen (nearly proportional to dry weight per unit projected area) can be compensated and thus measured.

1. *Human erythrocytes.* Red cells suspended in heparinized plasma were photographed with increasing amounts of compensation. A satisfactory picture of the shape of the cells was obtained from these "contour" photographs, together with refractometer measurements, and the mean cell volume agreed with that obtained from the haematocrit and cell count.

2. *Nucleoli from Asterias eggs.* Useful estimates of the solid content of nucleoli isolated by Dr. Vincent were obtained, but their precision was limited by the irregular vacuolation of these structures.

3. *Nuclei of Arbacia eggs.* The solid content of nuclei isolated by Dr. Tsuboi was estimated, and was found to confirm an observation by him on their specific gravity.

4. *Phascolosoma muscle.* Photographs were taken of muscle cells isolated by Dr. Prosser by maceration of the retractor muscles of the proboscis of *Phascolosoma.*

5. *Isolated myofibrils.* Fibrils obtained by treating glycerinated rabbit muscle in the Blendor were examined in collaboration with Dr. A. G. Szent-Györgyi. The extraction of various components of the striations by salt solutions was observed.

The effects of various acids on the receptors of the amphibian tongue. THOMAS N. JOHNSON.

Sensory impulses were recorded from the frog glossopharyngeal nerva. The impulses were excited by application of water, sea water, and mechanical stimulation to the sensory receptors in the tongue. Solutions of three acids, acetic, citric, and hydrochloric, were applied to the tongue, and the stimulation repeated. Buffered solutions of each acid at pH 6.0, 5.5, 4.5, and 3.5 were employed.

At pH 6.0, none of the acids affected the sensory discharges.

At pH 5.5, application for three minutes of acetic and of hydrochloric acid diminished the responses to all stimuli. After return from the acid to amphibian Ringer, the responses increased, but not to their original level. Further application of these acids completely and irreversibly abolished responses to water and sea water, but responses to touch remained.

At the same pH (5.5), citric acid had the reverse effect. The responses to all stimuli were increased for several minutes after application. The enhancement by citric acid is counteracted by the addition of acetic acid as well, which retains its effect of diminishing responses below the normal level.

At pH 4.5, all three acids diminish the responses, and a second application abolishes them.

At pH 3.5, all acids employed abolish responses to water and sea water, but responses to touch persist for as long as two hours.

It appears that moderate H ion concentrations diminish responses, and that high H ion concentration irreversilbly damages the sensory apparatus. Citrate ions increase the responses, probably by the precipitation of calcium ions, and this increase masks the depression by the H ions, provided that the H ion concentration is moderate. At the high H ion concentrations, the depression becomes predominant.

A study of an unidentified ultraviolet absorbing substance occurring in the tissues of marine animals. HERMAN M. KALCKAR, JACK L. STROMINGER AND NORMAN R. GEVIRTZ.

The mantle tissue and the foot muscle of *Busycon canaliculatum* were found to contain large amounts of a substance with maximum absorption at 272 mμ. The absorption spectrum does not change between pH O and 14 and is unaffected by treatment with sodium nitrite or by heating to about 200° C. in concentrated HCl. The compound was prepared by absorbing partially neutralized (pH 4 to 5) perchloric acid extracts of tissue on Norite and eluting with 50 per cent slightly ammoniacal ethanol. A further purification step by which all ninhydrin-positive material was removed consisted of saturation with Na_2CO_3, repeated extractions with 5 volumes of water-saturated butanol and finally returning the compound to aqueous solution by extraction with water. At this point an impurity can be precipitated with Reinecke salt at pH 1. The compound has the following properties: contains nitrogen; R_f in ammoniacal butanol 0.12, in 5 per cent KH_2PO_4 saturated with isoamyl alcohol 0.85; immobile on paper electrophoresis at pH 7.9; absorbed on Dowex-50-H^+ and eluted with 2 N HCl; not retained on Dowex-2-acetate even at pH 12. As judged by most of the above listed properties the same compound has been obtained from the muscle of lobster and crab, and also from the nerve tissue of crab and lobster and from squid axoplasm (*cf.* A. Shanes, 1951). The possible identity with or relationship to homarin, *i.e.*, N-methyl picolinic acid betaine (*cf.* F. Hoppe-Seyler, 1933), is under investigation.

The bacterial reduction of nitro compounds. JOSEPH R. MERKEL.

Bacteria were selected which could reduce nitrate to various levels including ammonia and gaseous nitrogen. The bacteria used included laboratory strains and strains isolated from infected marine specimens. All the strains had the ability to reduce nitrates to nitrites, and most of the cultures produced large amounts of ammonia and hydrogen. The production of hydrogen and ammonia tended to obscure any production of nitrogen.

Manometric studies with strain B of *Escherichia coli* and with a strain of *Proteus* (presumably, *vulgaris*) indicated that the production of ammonia from nitrate or nitrite involved the formic "hydrogenlyase" system. Addition of nitrate or nitrite to a phosphate-buffered medium (pH. 6.9) containing formate or glucose and inorganic salts, under anaerobic conditions, caused a reduction in the rate of hydrogen production which was equivalent to the amount of nitrate or nitrite converted to ammonia. The decrease in the rate of gas production may be a result of the hydrogenase activity of these cultures; however, it seems more likely that the reduction of the nitro compounds is linked more directly to the formic dehydrogenase system. In this system the reduction of nitrate is analogous to reduction by zinc and HCl, where the nascent hydrogen is picked up by an oxidized substance before molecular hydrogen is formed, rather than an activation of gaseous hydrogen.

A possible method which has been suggested for the formation of nitrogen by bacterial cultures (denitrification) is through the reaction of nitrous acid with amino compounds. It is conceivable, on the basis of the relative ease of reduction of aromatic nitro compounds, that this reaction may be mediated through aromatic nitro and amino compounds. Future experiments with active denitrifying cultures will be designed to test this hypothesis.

Nucleic acid and protein distribution in unfertilized and fertilized eggs of Arbacia. K. K. TSUBOI.

Homogenates of Arbacia eggs were subjected to successive centrifugal forces ranging from 250–100,000 × g and the resulting fractions examined for nucleic acids and protein (protein estimated by nitrogen, nucleic acids by u. v. absorption and phosphorus following appropriate fractionation).

Unfertilized eggs homogenized in isotonic, hyper- and hypotonic electrolyte solutions (KCl, NaCl, artificial sea water) and non-electrolytes (sucrose, dextrose) in the presence and absence of added chelating agents (citrate, Versene) were investigated. The presence of chelating agents in homogenates prepared in hypotonic electrolyte solutions and all non-electrolyte solutions resulted in a redistribution of the bulk of cytoplasmic pentose nucleic acid (PNA) from a form sedimentable only at very high centrifugal force (100,000 × g) to a relatively non-sedimentable state. The homogenization of eggs in stronger salt solutions ($> 0.4\ M$ KCl) only, resulted also in a "solubilization" of the PNA. Although some redistribution of protein was apparent in these studies, the extent was not comparable to that found for the PNA. PNA was readily sedimentable from homogenates prepared in artificial sea water. From these results it is concluded that the PNA in unfertilized eggs is present in submicroscopic particulates in combination with calcium and/or magnesium as a binding force in maintenance of the complex.

Experiments on fertilized Arbacia eggs are only of a preliminary nature due to difficulties encountered in preparing suitable homogenates from the embryos. Little evidence was found in preliminary experiments for the presence of extranuclear DNA within 20 hour embryos. A redistribution of protein and PNA appeared to be present within the 20-hour embryo, resulting in a much greater proportion of sedimentable protein at 100,000 × g and a more ready sedimentation of PNA within fractions obtained by less centrifugal force.

The apyrase activity of mantle muscle of Loligo pealii. G. W. DE VILLAFRANCA.

An investigation of the apyrase activity of squid mantle muscle was undertaken in an attempt to ascertain whether this muscle contained the two apyrases, *i.e.*, particulate and myosin, found in rat and locust muscle.

Squid mantle was extracted with distilled water, molar sucrose, or 0.05 M KCl. After the water or sucrose extraction the residue was re-extracted with 0.5 M KCl to recover the myosin proteins. Myosin was precipitated from the 0.5 M KCl extract by dilution with 6 volumes of distilled water and was re-precipitated once or twice. The apyrase reaction was carried out at 25° C. in 0.005 M KCl and 0.002 M ATP under conditions where the activity was directly proportional to the concentration of protein and to the time incubated. The myosin activity was maximal at pH 5.8 and 0.01–0.03 *M* $CaCl_2$ yielding Q_P values as high as 1700. $MgCl_2$ gave slight activation at concentrations of 0.01–0.05 *M* while $CaCl_2$, in optimal concentrations, gave 8–10 fold activation. A K_m of 4.07×10^{-4} was obtained.

Under the extraction conditions tried, the major portion of the total apyrase activity (60–80%) was recovered with the myosin fractions. Another 10% of the activity was recovered in material sedimented at 20,000 g which had the same pH optimum and ion activation as the myosin fractions. At least 70% of this activity, however, could be removed by 0.5 *M* KCl. It would seem that the apyrase of large particles from squid mantle is negligible or absent. Less than 2% of the total activity was found in fractions sedimenting at 100,000 g.

It appears, therefore, that squid mantle muscle has only one apyrase associated, apparently, with the myosin proteins. Preliminary experiments indicate that a similar situation exists in muscle from *Mustelus canis,* although here the apyrase is Mg^{++} activated.

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NEW OR NOTEWORTHY VAUCHERIAE FROM NEW ENGLAND SALT MARSHES

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The known *Vaucheria* flora of marine and brackish habitats in northeastern United States includes only four species, *V. thuretii* Woronin, *V. litorea* C. Agardh, *V. compacta* (Collins) Collins, and *V. piloboloides* Thuret. Apparently no additions have been made to this list since the publication of Taylor's Marine Algae of the Northeastern Coast of North America (1937). In the course of an ecological study of Great Pond, near Woods Hole, Massachusetts, winter and early spring collections by John T. Conover of salt-marsh and other coastal Vaucheriae have recently yielded three additional species which have not been previously reported from North America, and a fourth species which is new. Preserved material of these collections has been deposited in the Herbarium of the University of Michigan and in that of Professor William Randolph Taylor.

Section WORONINIA Solms-Laubach

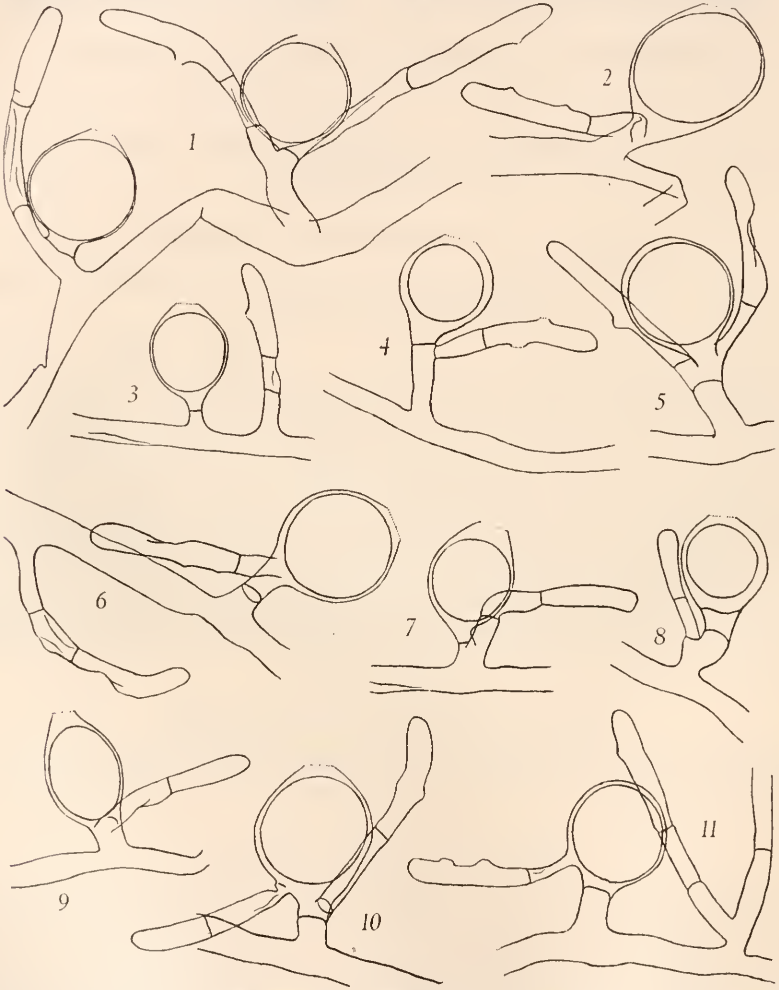
VAUCHERIA THURETII Woronin. *Specimens examined:* MASSACHUSETTS: East Shore Marsh, Great Pond, Falmouth, Barnstable Co., *Conover* 53-6, Jan. 31, 1953.

Section CONTORTAE Dangeard

V. ARCASSONENSIS Dangeard. While most of the other species listed in this paper are known to be present in many localities on the coast of Europe, no collections of this species appear to have been made since it was described from the region of Arcachon, France. The American material was fruiting abundantly in April and early May, 1953. It does not seem to differ significantly in any way from the collection reported by Dangeard (1939). *Specimens examined:* MAINE: North shore, Harraseeket River estuary near South Freeport, Yarmouth Co., *Conover* 53–1, April 24, 1953. MASSACHUSETTS: Southeast shore marsh, Great Pond, Falmouth, Barnstable Co., *Conover* 53–2, April 6, 1953; *idem, Conover* 53–3, May 19, 1953; West bank marsh, Great Pond, *Conover* 53–4, April 6, 1953; East shore marsh, Great Pond, *Conover* 53–5, May 11, 1953.

Section PILOBOLOIDEAE Walz em. Nordstedt

V. COMPACTA (Collins) Collins. This species was fruiting in the east shore marsh of Great Pond in January, 1953, mixed with *V. intermedia* Nordstedt and *V. thuretii.* It is reported by Taylor (1937) and has recently been identified by Christensen (1952) with the entity known in Europe as *V. sphaerospora* var. *dioica* Rosenvinge. *Specimens examined:* MASSACHUSETTS: East shore marsh, Great Pond, Falmouth, Barnstable Co., *Conover* 53-6, Jan. 31, 1953.



FIGURES 1–11. *Vaucheria intermedia, Conover* 53–11, from Great Pond, Massachusetts. Magnification approximately 160 ×. All figures drawn with the aid of a Spencer camera lucida.

V. CORONATA Nordstedt. This well-known European species has also been reported from Greenland (Collins, 1909). The material from Great Pond was fruiting abundantly from February through April, 1953, and was more plentiful here than any other species. The American specimens agree in all details with Nordstedt's description. *Specimens examined:* MAINE: North shore, Harraseeket River estuary, near South Freeport, *Conover* 53–7, April 24, 1953. MASSACHUSETTS: East shore marsh, Great Pond, near Falmouth, Barnstable Co., *Conover* 53–8, March 22, 1953; Southeast shore marsh, Great Pond, *Conover* 53–9, April 6, 1953; West bank marsh, Great Pond, *Conover* 53–10, April 6, 1953; Southwest shore of Perch Pond (northwest arm of Great Pond estuary), *Conover* 53–16, May 11, 1953.

V. INTERMEDIA Nordstedt (Figs. 1–11). This species is likewise well-known in Europe. It is characterized by relatively small dimensions throughout. The fruiting branch is short and in the American material bears one oogonium and usually one or two antheridia. The oogonium is more or less erect and opens by a wide, circular pore at its upper end. The oospore is usually spherical or sub-spherical, although it is sometimes ovoid (Fig. 9). The antheridium is cylindric and straight and opens by usually one to three lateral pores at the end of very short lateral tubes. It is always subtended by what appears to be a thin-walled hyaline supporting cell, but is probably only an empty space.[1] These two units, together with any portion of the thallus which may be in linear connection with them, can be called an antheridial filament. When borne on a bisexual fruiting branch, the antheridia are terminal as in the Racemose Vaucheriae; presumably they are formed well before the oogonia, a point which cannot be checked because of the absence of developmental stages in this material. The oogonium may develop from a lateral outgrowth of the lower portion of the fruiting branch, which is continuous with the vegetative filament; in this case the oogonium soon becomes cut off by a cross wall from the adjacent portion of the fruiting branch. The oogonium may, however, develop as an intercalary cell in a linear series which has the lower portion of the fruiting branch as its base and the antheridium at its distal end. Due to the unilateral, outward growth of the oogonium, however, the antheridium always appears to be in a lateral position when the oogonium is mature.

There are essentially four different ways in which the antheridial filament may be attached with reference to the oogonia:

1. On a separate (antheridial) branch of a vegetative filament, usually near an oogonial or bisexual fruiting branch (Figs. 3, 6).
2. Terminal on a bisexual fruiting branch, but apparently borne on a separate (antheridial) branch of the basal portion of the fruiting branch (Figs. 4, 8).
3. Terminal on a bisexual fruiting branch, but apparently subtending the oogonium, attached to its cylindric basal portion (Figs. 5, 6).
4. Terminal on a bisexual fruiting branch, but apparently a lateral offshoot of, or even surmounting, the oogonium, as a consequence of the lateral enlargement of the latter about a point more or less within the original axis of the fruiting branch (Figs. 10, 11).

Frequently the fruiting branch is itself branched, with a second antheridial filament borne as a branch of the first. When two antheridia are present on one fruiting branch, the oogonium generally lies somewhere between them (Figs. 1, 5, 10).

Vegetative diameter of the American material ranges from 14 μ to 35(–51) μ.

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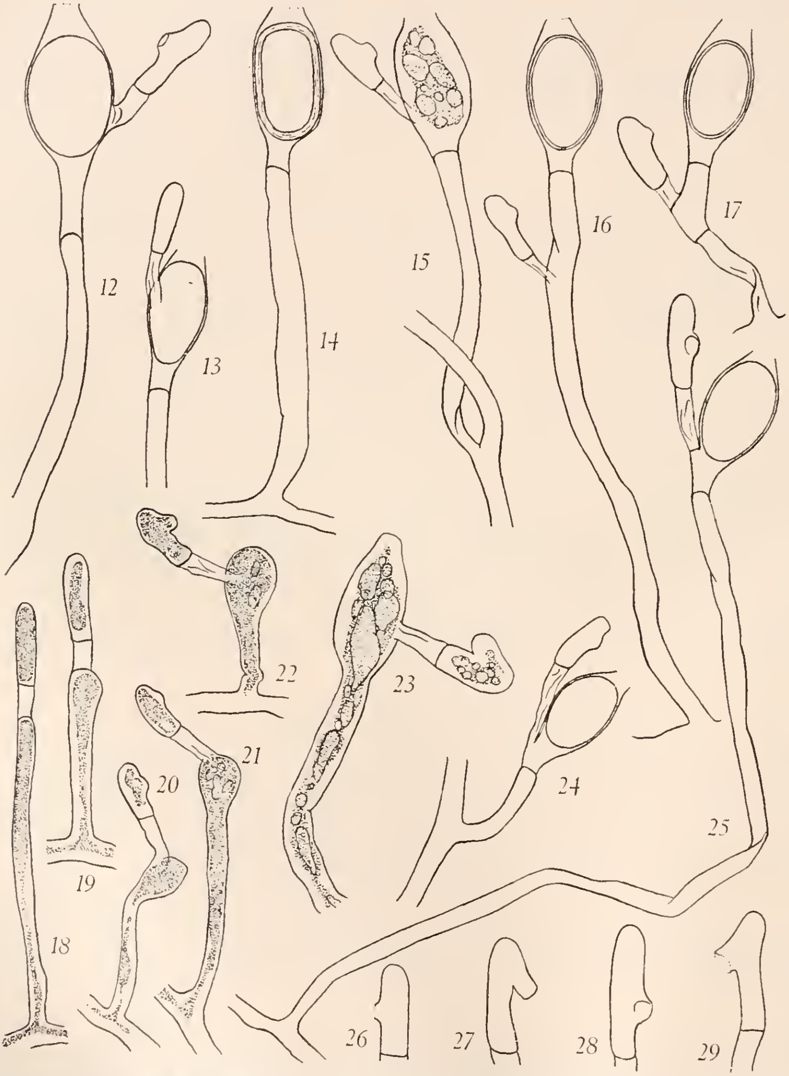
[1] Cf. Dangeard, 1939, with reference to *V. litorea.*

Antheridia measure 12–19(–34) × (51–)70–110(–124)μ. Oogonia measure 51–91 × 78–112 μ. The oospores, usually spherical, measure 52–94 μ in diameter. The length of the basal part of the fruiting branch, measured from the vegetative filament to the oogonial cross wall, varies from 11–76 μ. These measurements are in general agreement with those given by Nordstedt (1879) and Dangeard (1939).

This species was fruiting abundantly in Great Pond in January, 1953, mixed with *V. thuretii, V. compacta,* and *V. minuta* sp. nov. *Specimens examined:*



FIGURES 12–29. *Vaucheria minuta* sp. nov. *Conover* 53–12, type, from Great Pond, Massachusetts. Figures 12–17, 24, 25, mature or nearly mature bisexual fruiting branches. Figures 18–23, immature fruiting branches. Figures 26–29, antheridia. Magnification approximately 160 ×. Figures 12–25 drawn with the aid of a Spencer camera lucida.

MASSACHUSETTS: East shore marsh, Great Pond, Falmouth, Barnstable Co., *Conover* 53–11, Jan. 31, 1953.

Vaucheria minuta sp. nov. (Figs. 12–29).

Filamenta vegetativa 8.5–15(–17)μ diam.; oogonia antheridiaque in ramo fructifero longo portata, antheridiis terminalibus, per spatium vacuum celluliforme subtensis, oogoniis intercalaribus proxime infra hoc spatium enascentibus, aut oogoniis terminalibus, antheridiis nullis; antheridium ad oogonii maturi latus basimve plerumque affixum; rami fructiferi 195–820 μ long., antheridia cylindrica, 13.5–17 × 47–64 μ, e poro laterali unico, oogonium aspiciente, aperienta; oogonium symmetricale, ovatum ad cylindricum, 51–54(–64) × 102–125(–170)μ, e poro terminali erecto lato aperiens; oospora ovata ad cylindricam, 50–53(–62) × 68–92 μ, membrana oosporae maturae satis crassa.

Vegetative filaments 8.5–15(–17)μ in diameter; oogonia and antheridia borne on a long fruiting branch, the antheridia terminal, subtended by an empty cell-like space, the oogonia intercalary and arising immediately below this space; or the oogonia terminal and antheridia absent; at maturity of the oogonium, the antheridium is usually attached to the side or base of the oogonium; fruiting branches 195–820 μ in length; antheridia cylindrical, 13.5–17 × 47–64 μ, opening by a single lateral pore which faces the oogonium; oogonium symmetrical, ovoid to cylindric, 51–54 (–64) × 102–125(–170)μ, opening by a wide, erect terminal pore; oospore ovoid to cylindric, 50–53(–62) × 68–92 μ, with a relatively thick wall at maturity.

This species is distinguished from *V. intermedia* (1) by the extremely small size of its vegetative filaments, (2) by its relatively shorter antheridia, each bearing a single pore, (3) by the characteristic ovoid to cylindric oospores, which contrast markedly with the spherical oospores of *V. intermedia,* and (4) by the very much elongated fruiting branches, quite unlike the short branches of *V. intermedia.*

It is, however, clearly related to the latter species as well as to *V. sphaerospora* Nordstedt since these are the only species known to possess an antheridial filament which may surmount and have its point of attachment directly on the oogonium. Because of this peculiarity of the antheridial filament, which appears frequently as if it were an epiphyte on the oogonium, these three species are to be considered as set somewhat apart from the remaining species of the Section Piloboloideae.

The development of the antheridium and oogonium has been studied in preserved material by the comparative observation of immature fruiting branches of different ages (Figs. 18–23). It appears likely that the antheridium is always terminal on a bisexual fruiting branch, although this point must remain in doubt until development of the fruiting branch can be followed in living material. Several fruiting branches were found in which the filament immediately below the antheridial supporting cell or space was only slightly enlarged (Figs. 19–21). Later stages show the antheridial filament clearly pushed aside and tipped from its apical, erect position by the unilateral and upward enlargement of the young oogonium (Figs. 22–23). A similar series of developing fruiting branches has been illustrated for *V. sphaerospora* by Nordstedt (1879). Filaments lacking an enlargement, such as the one shown in Figure 18, are observed rather commonly; actually there is no assurance that these are destined to form bisexual fruiting branches—they may merely represent antheridial branches which will never form an oogonium.

V. minuta is distinctive and unlike other Vaucheriae in at least two ways: (1) its vegetative filaments are smaller in diameter than those of any other known species, and (2) it has cylindric antheridia which open by a single lateral pore borne at the end of a short tube. Descriptions of all of the other members of the Piloboloideae indicate that the antheridia have a variable number of pores, usually 1–2 or 1–3. In *V. minuta* the single pore with its tube usually faces the oogonium and frequently is directed somewhat toward the base of the antheridial filament (Figs. 23, 27, 29). Occasionally this lateral tube is so well developed as to give the whole antheridium the appearance of being reflexed (Fig. 23). As in *V. intermedia,* the antheridium is subtended by a sterile supporting cell or empty space which is thin walled and lacks protoplasm. Likewise as in *V. intermedia,* the antheridial filament is highly variable in position, and may be found in the same series of situations listed above for *V. intermedia;* however, in this connection it may be mentioned that no unquestioned short antheridial branches have been found on vegetative filaments of *V. minuta* (position 1). All of the isolated "antheridial" branches are hence open to the suspicion of being immature fruiting branches as yet lacking the oogonial enlargement.

Fruiting branches which lack antheridia (Fig. 14) are common. It is impossible to determine from preserved material whether the "oogonia" formed on these structures contain oospores or parthenospores. Usually these spores are more distinctly cylindrical, and thicker walled, than are the undoubted oospores borne on bisexual fruiting branches.

V. minuta was found fruiting in Great Pond in March and May, 1953, and sparsely fruiting in the Harraseeket River estuary in April. Its habitats range from submerged areas of relatively high salinity (29.5–30.2 parts per thousand) to emergent situations subject to wide range in salt concentration. At the time of fruiting, the algae were covered by water of salinity ranging from 26.0 to 28.0 parts per thousand. *Specimens examined:* MAINE: North shore, Harraseeket River estuary, near South Freeport, Yarmouth Co., *Conover* 53–17, April 24, 1953. MASSACHUSETTS: TYPE in Herbarium of the University of Michigan; east shore marsh, Great Pond, Falmouth, Barnstable Co., *Conover* 53–12, March 22, 1953; *idem, Conover* 53–14, Jan. 31, 1953; *idem, Conover* 53–15, May 11, 1953.

ECOLOGICAL REMARKS

The Vaucheria species here discussed have been collected near spring and neap high tide levels and appear to be confined to this relatively narrow zone on the borders of estuaries or in well drained brackish marshes which are arms of the sea at high tide. These algae regularly grow as a mat of 2–4 *Vaucheria* species mingled with Myxophyceae and Chlorophyceae of brackish water. This mat commonly occurs in scattered, moderately dense to sparse stands of *Juncus, Spartina,* or various other graminioids, covering their rhizomes and the intervening soil surface over an area which may be as much as twenty-four square meters in extent. When the water from the estuary does cover the algal mats, it ranges from 12.0 to 27.0 parts per thousand in salinity. Due to strong onshore winds in late fall of 1952 and early spring of 1953, however, the *Vaucheria* vegetation was covered continuously at these times for a period of several days by water of somewhat higher salinity (26.0–28.0 parts per thousand). The mats of *Vaucheria* may be covered by brackish water at times of spring and neap tides, but the water level of the usual diurnal tides apparently does not reach them. During such periods when the algal zone is emergent, the *Vaucheria* mats are subject to fresh runoff. They are hence exposed regularly to wide variations in salinity, from that of fresh water to very nearly the concentration of the sea.

Partial shade seems to be necessary for the growth of these *Vaucheria* species. Although they grow commonly over sandy to peaty soil at the base of graminioid plants, they are not found where these phanerogams grow densely and shade is heavy. Neither are they found in unshaded areas.

While the seasons at which these species were found in fruit varied from species to species, none were found in fruit at temperatures above 19.0 degrees Centigrade. Temperatures which favor fruiting range from 0.0 to 18.5. No fruiting *Vaucheria* was found in any of the numerous collections made from the same meter square quadrats in the warmer months from late May through November.

In view of the relative thoroughness with which the Woods Hole area has been explored by phycologists in summer, it would appear that most of these species have escaped detection because of a fruiting period which is confined to the winter months. The near-absence of fruiting material in all *Vaucheria* collections made in Great Pond in the period from May through July, 1953 continues to bear out this supposition.

The authors wish to thank Professor William Randolph Taylor for the advice he has given during the progress of this work and for his critical reading of the manuscript.

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CUTANEOUS AND TRACHEAL RESPIRATION IN THE PHORMIA LARVA

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The larvae of most calypterate flies, of which *Phormia regina* Meig. is an example, have a relatively simple amphipneustic tracheal system consisting basically of a pair of main longitudinal trunks opening to the exterior through one pair of anterior and one pair of posterior spiracles (Fig. 1A, 1B). The anterior and posterior spiracles are strikingly different in structure, but no measurements of their relative importance in the over-all gas exchange of the larva have been reported previously. Similarly, except for the work of Fraenkel and Herford (1938) on *Calliphora,* there are practically no quantitative data on the relative magnitudes of oxygen uptake through the skin and by way of the tracheal system. The *Phormia* larva has proved to be excellent material on which to investigate these problems, particularly because it has remarkable tolerance to radical experimentation: for example, it will live for many hours with either or both ends of the tracheal system closed off by ligatures. Some of the results obtained have been published in abstract (Buck and Keister, 1950a, 1950b; 1953).

The assistance of Mary Ann Dawson in part of this work is acknowledged with pleasure.

MATERIAL AND METHODS

Third instars of *Phormia regina* were collected at about the time of emptying of the digestive tract preparatory to pupation (about 5 days after egg laying, at 25° C.). Culture methods, techniques for holding and ligating the larvae and procedures for stimulating respiration with DDT have been described previously (Buck, Keister and Posner, 1952). In all cases "ligated" refers to larvae with ligatures cutting off one or both pairs of spiracles, whereas "deganglionated" refers to larvae with a ligature just posterior to the brain (which also, of course, closes the tracheae at that point). "Hypoxia" is used to mean a condition in which some aerobic respiration is occurring, but in which oxygen uptake is limited by pO_2.

In applying ligatures there is no direct test of whether the tracheae are actually pinched completely shut, and since the logic of the whole investigation depended on this being true, the ligatures were made tighter than was probably necessary. As previously reported, this not infrequently resulted in one or both of the tracheal trunks actually being severed, particularly in ligating off the anterior spiracles. Less trouble was experienced with deganglionations, since the ligature needed to be only tight enough to prevent leakage when the head end was removed. Whenever blood entered a cut trachea the larva was discarded. Usually, however, the trachea was quickly sealed off at the break point by a small melanotic plug or cap formed at the interface between blood and intratracheal gas. Since the trachea remained *in situ* and since there was no other visible internal damage—*e.g.*, to the gut—such larvae appeared to be physiologically equivalent to larvae in which the tracheae were not cut, and might even be considered to have the advantage of giving visible proof that no gas could enter the tracheal system through the spiracle in question. Since also, in an extensive series of tests, larvae with cut trunks behaved and respired indistinguishably from those with intact ligated trunks—in sharp distinction to the moribund and severely hypoxic larvae with flooded tracheae—such larvae, representing about 25% of the anteriorly ligated, 10% of the posteriorly ligated, and 10% of the deganglionated animals, were used in the respirometry.

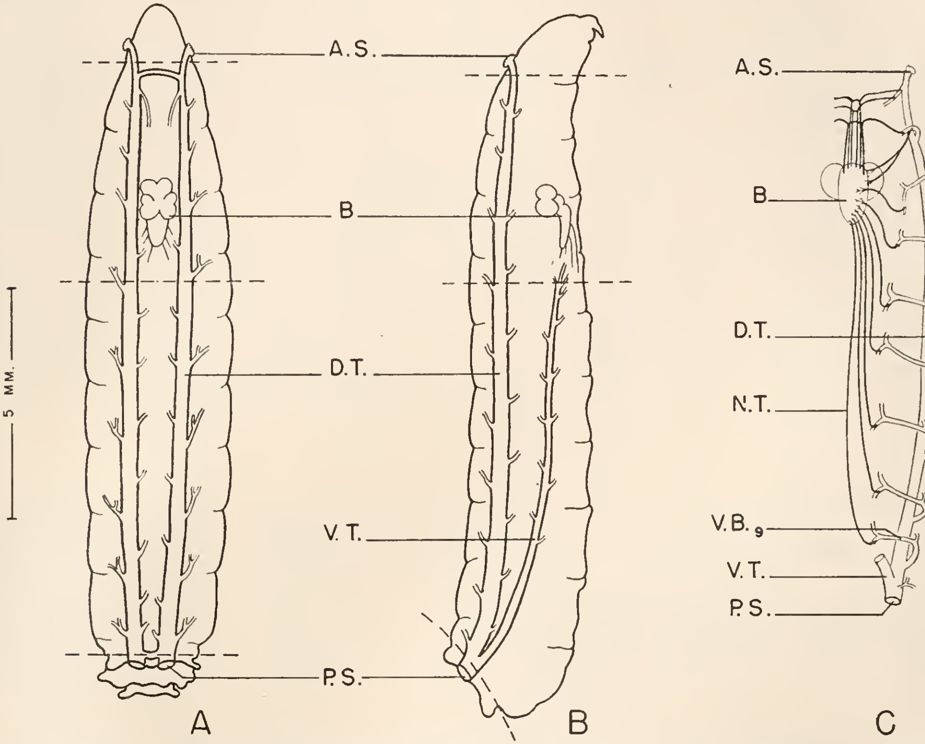

FIGURE 1. A and B, dorsal and lateral diagrammatic views of tracheal system of third stage larva of *Phormia regina* Meig. C, diagram (at slightly less magnification than A and B) of ventral view of neural tracheae (solid black), the dorsal trunk being displaced to the apparent right. A.S. = anterior spiracle; B = brain; D.T. = dorsal trunk; N.T. = neural trachea; P.S. = posterior spiracle; V.B. = ventral branch of dorsal trunk; V.T. = visceral trunk. Dotted lines indicate positions of ligatures.

Improvements made in our technique for administering "light" doses of DDT (Buck, Keister and Posner, 1952) involved using only the minimum clamp pressure necessary to hold the larva (which eliminated leakage due to back pressure), injecting always through the center slit of the left posterior spiracle, and selecting only larvae having DDT solution filling at least the left medial neural trachea (Fig. 1C). With the dose used (approximately 0.6 μL per 60 mg. larva, corresponding to 50 mg./kg. live weight) all larvae were incapable of locomotion at 3½ and at 8

TABLE I

Mean oxygen uptake of Phormia larvae in µL/mg. live wt./hr., with standard errors. Figures in brackets are for Calliphora at 27° from Fraenkel and Herford, 1938. Inter-group comparisons should be made between same batch numbers (see text). No group of larvae was used for more than one run.

		Batch	Unligated			Anterior ligation			Posterior ligation			Double ligation		
			No. of larvae	Mean uptake	% of control	No. of larvae	Mean uptake	% of control	No. of larvae	Mean uptake	% of control	No. of larvae	Mean uptake	% of control
Unpoisoned	Air	292	18	1.15±.03					17	0.69±.01	60			
		373	17	0.92±.08								17	0.09±.003	10
		377	20	0.99±.02								19	0.09±.005	9
		379	22	0.95±.02										
		383	18	0.67±.02		29	0.95±.04	141						
		384	18	1.04±.07										
		385	17	1.07±.06					16	0.71±.03*	66			
		387	20	0.92±.02		16	1.30±.08*	141						
		388	26	1.07±.03										
		446	54	1.09±.02										
			[4**	0.72]		[5**	0.67	93]				[5**	0.15	21]
	Oxygen	273–4	10	1.24±.05										
		292	19	1.11±.04	97				17	1.27±.06	110			
		373										16	0.37±.02	39
		377										21	0.36±.01	36
		383				28	1.10±.07	164	18	1.32±.05*	123			
		385												
		387				16	1.48±.12*	161						
		388	25	1.10±.02	103							[5**	0.38]	
Poisoned	Air	273–4	20	2.55±.08	206									
		373										18	0.09±.004	10
		377										20	0.10±.01	10
		379				19	2.50±.05	263	21	1.20±.04	126			
		446	33	2.74±.06	250	35	2.38±.05	218	23	1.04±.02	96			
	Oxygen	273–4	18	3.45±.06	278									
		373	17	3.21±.05	349							18	0.35±.01	38
		377	20	3.58±.12	362							22	0.34±.01	34
		379	21	3.43±.06	361	21	3.37±.08	355	21	2.39±.04	251			
		384	30	3.48±.04	341				22	2.37±.05*	228			
		446	32	3.46±.07	317	37	3.21±.06	294	27	2.42±.03	222			

* Another run also made, with almost identical results, on larvae ligated immediately after poisoning and compared with separate contemporaneous controls. ** Indicates number of flasks, each containing about 10 larvae, run by Fraenkel and Herford.

hours after poisoning (tested on wet filter paper) but were often capable of locomoting at 24 hours, and were usually successful in forming apparently normal puparia (although few succeeded in emerging as adult flies).

Oxygen uptake was measured individually in 15-ml. Warburg flasks, using cylindrical wire screens to keep the control larvae out of the KOH, as described for adult flies by Buck and Keister (1949). The oxygen uptakes given in the table are means over a two-hour plateau period, usually between 5½ and 8 hours after the start of poisoning. Manometers were shaken at about 110 cycles per minute to aid temperature control.

The schedule of procedures carried out by two workers on the 40-odd larvae used in an experiment averaged about as follows: hours 0–1, poisoning by intratracheal injection of 5% DDT in kerosene and checking dosage microscopically; hours 3½–4, weighing and setting up control and unligated poisoned larvae (the latter being first tested for inability to locomote) in Warburg flasks; hours 3½–4½, ligating, inspecting and setting up control and poisoned larvae; hours 4½–5, flushing certain flasks with oxygen (3½–4 L. passed through each flask in 5 min.), leaving others in air, and equilibration; hours 5–8, recording oxygen uptake at half-hour intervals, of which the first period was discarded; hours 8–8½, re-inspecting ligated larvae and testing unligated poisoned larvae for inability to locomote. The present results include only gas phase respiration: data for respiration in water will be reported elsewhere.

Unless otherwise noted, all experiments were made at 25° C. Statistical significance was assessed by t test, using the 2% level.

Results

Oxygen uptakes from gaseous air or oxygen of larvae under various conditions are given in Table I; those of unpoisoned larvae in the upper half, those of larvae with DDT-stimulated respiration in the lower half. As in previous work, the variability in the material made it desirable to give data from a number of different batches for each category of larva, each with its own control, and to draw all conclusions from statistical analysis of intra-batch comparisons. To facilitate such comparisons, mean uptake rates are given both in μL/mg. live wt./hr. and as percentages of that of normal controls for the particular batch of larvae. Some groups seem to be more homogeneous when compared on one basis (*e.g.,* unligated, poisoned in oxygen) and some on the other (*e.g.,* anterior ligated, unpoisoned).

A. Cutaneous respiration

In gaseous air the oxygen uptake of doubly ligated larvae was approximately 10% of that in unligated controls (Table I). It is thus clear that larvae in which spiracular pathways of oxygen entry have been eliminated must become severely hypoxic.

In doubly ligated larvae poisoned intratracheally with DDT, oxygen uptake from air (10%) was no higher than that of doubly ligated unpoisoned larvae, in striking contrast to the great increase seen in poisoned unligated or once-ligated larvae in gaseous air or oxygen (Buck, Keister and Posner, 1952, and below). This, therefore, seems to indicate that, regardless of the potential metabolic rate, the maximum amount of oxygen which can be taken in through the skin from air is of the order of 10% of the normal uptake. It can also be concluded that the presence of DDT in the body does not alter cutaneous permeability to oxygen.

Cutaneous uptake from oxygen was only of the order of four times that in air (39% vs. 10% in both unpoisoned and poisoned larvae). It was not increased by suspending the (doubly-ligated) larvae from the necks of the manometers, so that they swung back and forth inside the flasks as the manometers shook. This indicates that access to environmental oxygen is not the factor preventing the respiration from increasing linearly with pO_2.

As pointed out by Fraenkel and Herford (1938), the severe hypoxia in larvae restricted to cutaneous respiration undoubtedly means that the internal pO_2 is lower than in normal larvae respiring in air, and the trans-cutaneous oxygen gradient correspondingly steeper. This indicates, therefore, that the observed cutaneous respiration (10%) is higher than in normal larvae. Estimates of the proportion of oxygen taken in through the skin in normal larvae, and of the internal pO_2 in larvae respiring only through the skin will be given in the Discussion.

B. Relative contributions of anterior and posterior spiracles to total oxygen uptake (*Table* I)

The oxygen uptake from air of unpoisoned larvae with only the posterior spiracles functional (anterior spiracles tied off) was about 40% higher than that of normal unligated larvae, due, presumably, to increased activity induced by the ligature. The uptake from air of larvae with the anterior spiracles, alone, functional was only 60–65% of what is required in normal metabolism. In both types of ligation, of course, some oxygen entered also through the skin, but since the amount was presumably proportional to the environment-tissue gradient it could not have exceeded, and probably was much less than, the amount passing through the skin of the larva with all spiracles blocked (10% of control). This indicates, therefore, that in unpoisoned larvae the posterior spiracles by themselves can pass at least as much air as is normally required, whereas the anterior spiracles by themselves cannot.

The conclusion as to the relative "capacities" of the two pairs of spiracles is supported qualitatively by the facts that (a) normal unligated larvae in osmium tetroxide vapor blacken first and most intensely at the posterior end, and (b) pupating larvae in which the anterior spiracles have been ligated off darken evenly and at normal speed, whereas those with the posterior spiracles, alone, ligated off, darken slowly and unevenly, starting at the front end. Fraenkel (1935) obtained the same effect in *Calliphora* by immersing alternate ends of the larva in oil. Additional more tenuous pieces of circumstantial evidence which are at least compatible with the subordination of the anterior to the posterior spiracles are: (c) in larvae immersed in kerosene, the oil invariably enters the tracheal system through the posterior spiracles; (d) in larvae held under water and pinched at one end or the other in such a way as to close one pair of spiracles, gas can be driven out of the posterior spiracles with relative ease, but out of the anterior spiracles only with considerable difficulty. A necessarily smaller gas volume passed by the anterior spiracles can perhaps also be deduced from the anatomical facts that (e) the diameter of the main tracheal trunk at the posterior spiracles is about 285 μ, whereas at the anterior spiracles it is only 135 μ, and (f) the only tracheal supply to the digestive tract comes off the main trunks at the extreme posterior end (visceral trunk, Fig. 1B), thus necessitating a long detour for gas supplied via the anterior spiracles.

C. *Effects of DDT and of oxygen on oxygen uptake in unligated and once-ligated larvae* (*Table* I)

After administration of DDT, oxygen uptake from air increased markedly in both anteriorly and posteriorly ligated larvae, and in unligated larvae. This is consistent with the increase in muscular activity characteristic of DDT stimulation (*cf.* Buck, Keister and Posner, 1952), which lowers the internal pO_2 and increases the environment-tissue oxygen gradient.

As set forth in Table I, normal larvae took up oxygen at the same rate from pure oxygen as from air, showing that oxygen is neither limiting nor stimulating to normal respiration in air. In all other groups, both poisoned and unpoisoned, oxygen uptake from oxygen was significantly higher than from air. As applied to the unpoisoned anteriorly ligated larvae this finding is perhaps a little surprising since

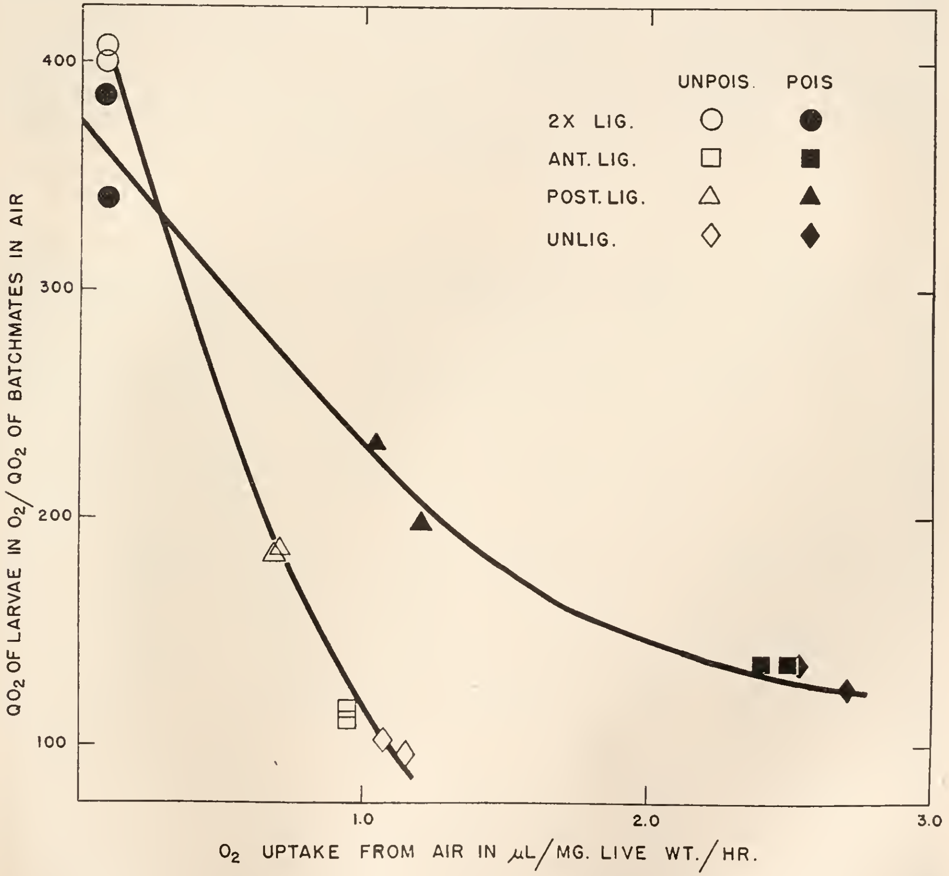

FIGURE 2. Relation between the rate of O_2 uptake from air by larvae with various types of respiratory restrictions (abscissae) and the degree (percentage) by which this is surpassed by the uptake of similar larvae in pure oxygen (ordinates). Data from Table I.

it indicates that even the stimulation due to the ligature makes the larva mildly hypoxic, and that the posterior spiracles alone, though adequate for control respiration, are only just so.

The metabolic stimulation due to DDT is apparently so great that even with four times as much oxygen available neither pair of spiracles by itself is adequate.[1] As a matter of fact, even the unligated poisoned larvae in oxygen might prove to be hypoxic—i.e., capable of a higher rate of oxygen uptake—if tested in pure oxygen at greater than atmospheric pressure.

It is understandable that the absolute respiratory rates of the poisoned larvae, both in air and in oxygen, should be in the order unligated > anteriorly ligated > posteriorly ligated > doubly ligated, since this reflects the order of the "capacities" of the available pathways of oxygen entry. On the other hand, the fact that the *percentage* increases in uptake shown by the various groups in oxygen as compared with air are in the reverse order in both unpoisoned and poisoned larvae is consistent with the expected order of severity of hypoxia. Thus the most hypoxic groups, the doubly ligated larvae, take up four times as much oxygen from pure oxygen as batchmates do from air, whereas the poisoned unligated larvae, which have all their normal respiratory avenues intact, show only about a 30% increase. The non-linearity of the *increase* in respiration with five-fold increase in pO_2 is well shown in Figure 2. Though unexplained, the curious inability of the poisoned singly ligated larvae to take up as much oxygen from oxygen as the unligated poisoned larvae (*i.e.*, to fully eliminate hypoxia) deserves attention.

Discussion

Other things being equal, it might be expected that the oxygen uptakes of the 60-mg. *Phormia* larva and the morphologically similar 80-mg. larva of *Calliphora* would be of the same order of magnitude. As shown in Table I this expectation is borne out fairly well for most of the comparable types of experiment in spite of the considerable differences in experimental techniques. One minor discrepancy is that Fraenkel and Herford (1938) reported uptakes from air slightly below normal in *Calliphora* larvae with the anterior spiracles ligated off, whereas we observed in *Phormia* an uptake about 40% above the control, which we attribute to the greater activity caused by the irritation of the ligature. Another difference is in the apparent proportion which cutaneous respiration forms of the total oxygen uptake from air (21% in *Calliphora;* 10% in *Phormia*), which is especially surprising in view of the fact that in *Calliphora* respiration was measured on samples of 10 larvae simultaneously, where constant mutual contact stimulation might have engendered an artificially high level of control activity. Possibly the difference is due to Fraenkel and Herford's having followed respiration for only an hour, a period which, in *Phormia,* is marked by violent struggling of the doubly ligated larvae, whereas all our calculations represent the means of the uptakes over a two-hour period several hours after the start of the experiment. However, there may well be a real interspecific difference in cutaneous permeability to oxygen, particularly in view of the difference in thickness of the cuticles and in reaction to paraffin-alcohol mixtures (Fraenkel, personal communication).

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[1] In batch 379 the O_2 uptake of unligated poisoned larvae in oxygen was not significantly higher than that of the anteriorly ligated poisoned larvae in oxygen (3.43 vs. 3.37), but the difference between the corresponding groups in batch 446 is so great that a highly significant difference ($p = < 0.01$) is obtained even if the two batches are lumped. It is evident that the anteriorly ligated larvae in oxygen are able to take in almost enough oxygen to avoid hypoxia.

Fraenkel and Herford attempted to circumvent the effect of voluntary activity by measurements on deganglionated larvae. These respired at a rate which was about half that of normal controls and which was assumed to be the basal rate. They found that the oxygen uptake of deganglionated larvae with the hind spiracles also ligated off was about one quarter of that of deganglionated larvae with functional rear spiracles (thus one-eighth that of normal larvae). As already pointed out, this would actually correspond, in normal (non-hypoxic) controls, to much less than an eighth (12%) of the total uptake. By indirect methods Fraenkel and Herford estimated that the true cutaneous respiration is about 10% of the basal (hence 5% of the normal control). Buck, Keister and Posner (1952) found a drop of 32% in the oxygen uptake of deganglionated *Phormia* larvae at 25° in one batch, and a nonsignificant drop of 7% in another. In check experiments for the present paper, each involving 40–50 larvae, we found a nonsignificant drop of 4% and a nonsignificant increase of 3% at 25° and a significant drop of 16% at 30°. Since Fraenkel and Herford did not check their larvae for leakage of blood into the tracheae, which occurs rather frequently in *Phormia,* it is possible that the presumed basal rate in *Calliphora* is too low because it represents an average of the uptakes of larvae with flooded tracheae (and hence respiring at the very low rate of twice-ligated larvae), and others with rates close to that of unligated controls.

The increased cutaneous respiration in doubly ligated larvae in oxygen (Table I) is the expected response of these severely hypoxic animals to increased environmental pO_2. The unchanged cutaneous respiration after poisoning is therefore especially significant, not only because it shows that DDT has not affected cutaneous permeability to oxygen, but because it indicates that DDT has not significantly affected the oxygen gradient between environment and tissues (assuming that static diffusion is the mechanism of gas transport across the integument). The simplest explanation of the non-effect of DDT is that the internal pO_2 is already zero even in the unpoisoned doubly ligated larva, and oxygen is hence already penetrating the integument at its maximum rate for the oxygen gradient of about 136 mm. Hg, which obtains in the Warburg flask at 25°. This is what Fraenkel and Herford (1938) had postulated for doubly ligated *Calliphora* larvae, on the basis of their work with varied pO_2. As a matter of fact, the equality of oxygen uptake in unpoisoned and poisoned doubly ligated *Phormia* larvae in *oxygen* (Table I) shows that even with a trans-cutaneous gradient of 737 mm. the tissue pO_2 is essentially zero, indicating *a fortiori* that it must be zero in such larvae respiring air.

By making use of the oxygen uptakes measured at the known gradients, the permeability of the integument to oxygen can be calculated. Thus, using the mean total skin area of 8 doubly ligated 60-mg. larvae (85 mm.^{2}), the permeability for air respiration (0.09 μL/mg. live wt./hr.) is 0.0056 μL/mm.^{2}/min./atm., and the value for oxygen 0.0042. A. G. Richards (personal communication) found good agreement between cutaneous permeability calculated from Warburg measurements on two doubly ligated larvae of *Sarcophaga bullata* in O_2 (the absolute values being about the same as in *Phormia*) and that calculated from oxygen electrode measurements on the isolated cuticles of the same larvae, using O_2-saturated water on one side and N_2-saturated water on the other. This supports the suggestion that the tissue pO_2 in doubly ligated larvae quickly falls to zero and that the recorded rate for cuticular penetration is indeed maximal.

Assuming that neither DDT nor oxygen causes a physical change in the pathways of oxygen entry, it seems reasonable to postulate that if oxygen uptake via the three possible routes (skin, anterior spiracles, posterior spiracles) could be measured in larvae in which the internal pO_2 was the same, the ratio skin : ant. : post. would be the same as in the normal larva even though the internal pO_2 in the experimental animals was different from the normal. In such circumstances one would expect the following arithmetical relation to hold: total uptake (*i.e.,* unligated larva) = (cutaneous uptake) + (uptake of anteriorly ligated larva minus cutaneous uptake) + (uptake of posteriorly ligated larva minus cutaneous uptake). In the present series of experiments it seems clear that a strict quantitative separation of the avenues of oxygen entry is not possible, because the ligating which is necessary to separate the three pathways causes different degrees of hypoxia and hence modifies the inwardly directed oxygen gradients. This is indicated, for example, by the relatively poor fit obtained in substituting, in the above equation, the oxygen uptakes for the most severely hypoxic groups (poisoned, in air) of batch 446, using any value up to 0.1 for the small cutaneous correction factors (x_1, x_2, x_3):

$$2.74 = x_1 + (2.36 - x_2) + (1.04 - x_3).$$

A rigorous budget could presumably be made out either (1) by subjecting the four classes of larvae to reduced pO_2 and comparing their oxygen uptakes at the highest respective pO_2s at which DDT had no stimulating effect (*i.e.,* reducing all groups to zero internal pO_2) or (2) by comparing the uptake from air of each group at the highest temperature at which flushing with pure oxygen had no effect (*i.e.,* lowering, in most cases, the temperature until the metabolic requirements could be met by the amount of oxygen which could enter through the restricted avenues). Experiments along both the lines suggested above are under way, but for present purposes useful approximations can be derived from the data already available (Table I). For example, if we average the uptakes of the two batches in each of the ligated poisoned groups in air we get, neglecting the corrections for cutaneous uptake, a cutaneous : anterior : posterior ratio of 0.095 : 1.12 : 2.44, or approximately 1 : 12 : 26. As already discussed, we know that the cutaneous contribution must normally be much less than in doubly ligated larvae, but this at least shows us at once that it cannot be *more* than 1/39 (2.5%) of the total. Furthermore, although the indicated uptake through the anterior spiracles alone should be proportionately too high, both because the posteriorly ligated animal is more hypoxic than the anteriorly ligated and because of the irritating effect of the ligature, an A : P ratio of 12 : 26 or 1 : 2 is probably roughly correct. This is indicated by the facts (1) that the A : P ratio is approximately the same in both unpoisoned and poisoned larvae in air; (2) that the percentage increase in respiration in oxygen as compared with air in the poisoned anteriorly ligated larvae (130%) is close enough to that in the poisoned posteriorly ligated larvae (210%) to indicate that their hypoxias are at least of the same order of magnitude; and (3) because the observed uptake through the anterior spiracles is undoubtedly lowered, as compared with normal, because the ligature cuts off the visceral tracheal trunks from the main longitudinal trunks at the same time it closes the posterior spiracles (Fig. 1). Furthermore, in experiments in which oxygen intakes by the two ends of the body were measured separately in normal control larvae stuck through a rubber membrane, an A : P ratio of approximately 2 : 3 was observed (Buck and Keister, unpublished data).

The present evidence of the subordinate physiological position of the anterior spiracles agrees also with opinions of Strasburger (1935) on *Drosophila* and Levenbook (1951) on *Gastrophilus.*

None of the evidence on the relative "capacities" of the two pairs of spiracles bears on the question of whether gas transport is via diffusion or ventilation. This problem will be dealt with elsewhere.

SUMMARY

1. In the *Phormia regina* larva oxygen uptake from air at 25° of larvae with both pairs of spiracles ligated off is about 0.09 μL/mg. live wt./hr. (10% of normal respiration) and increases four-fold in oxygen. This is the maximum cutaneous uptake possible from air.

2. The posterior pair of spiracles by itself can admit enough oxygen from air to supply normal needs.

3. On the basis of reciprocal ligation experiments, the anterior spiracles have about half the "capacity" of the posterior spiracles for admitting oxygen into the tracheal system, and can, by themselves, admit about 60% of normal needs.

4. The internal pO_2 in larvae with both pairs of spiracles ligated is zero, even in an atmosphere of pure oxygen.

5. In normal (non-hypoxic) larvae, cutaneous respiration accounts for less than 2.5% of the total oxygen uptake.

6. The permeability of the integument to oxygen is of the order of 0.005 μL/mm.^{2}/min./atm.

7. DDT does not affect cutaneous permeability to oxygen.

8. DDT-poisoned larvae cannot obtain enough oxygen from air even with all spiracles and skin functional, the hypoxia increasing nonlinearly in the order unligated $<$ anteriorly ligated $<$ posteriorly ligated $<$ doubly ligated. Even in pure oxygen the larvae are hypoxic if either pair of spiracles is cut off.

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THE CORRELATION OF DESOXYRIBONUCLEIC ACID SYNTHESIS AND THE RATE OF RESPIRATION IN THE SEA URCHIN EMBRYO[1]

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Measurements of the rate of respiration of sea urchin eggs from fertilization through early development have been reported by Warburg (1915), Gray (1925, 1926), Rapkine (1927), Ephrussi (1933), Tyler (1936b), Lindahl (1939), Horowitz (1940), Hutchens *et al.* (1942), Borei (1948), and others. In all experiments the rate of oxygen uptake is shown to increase during development. A number of attempts have been made in the past to learn about factors controlling, or related to, this increase. It seems not to be related closely to the increase in total nuclear volume (Godlewski, 1908), the increase in the number of cells of the embryo (Warburg, 1915; Tyler, 1936a), nor to the increase in the rate of cleavage of the cells of the embryo (Gray, 1926; Tyler, 1936b).

An experiment that suggests a possible relation between the rate of respiration and the content of desoxyribonucleic acid (DNA) was done by Brachet (1938). The oxygen consumption of fertilized eggs of *Chaetopterus* as well as eggs activated by KCl was measured and the respiration of the KCl-activated eggs, which differentiate without cleavage, was found to increase at a much slower rate than that of the fertilized eggs. It was found that the larvae from the KCl-activated eggs contained only 30% of the DNA present in the fertilized eggs several hours after the activation or fertilization. Brachet's interpretation was that (p. 97) "both the reduced oxygen uptake and the slower development are linked together; such a conclusion could support Tyler's opinion that part of the energy available in the egg is needed for the growth and differentiation processes taking place during development." Similarly, Tyler and Horowitz (1938) concluded, from an analysis of the effects of phenylurethane on respiratory rates of *Urechis* embryos, that the normal rise in rate of respiration is probably connected with nuclear division.

The purpose of the work recorded here has been to determine whether a correlation does exist between the rate of oxygen consumption and the DNA content of sea urchin embryos. If such a relationship were found, another conclusion, alternative to Brachet's, could be drawn: it would be possible that DNA, as a constituent of the genome of the cell, is limiting the production or activation of respiratory enzymes which would be responsible for the rate of oxygen consumption.

This problem was undertaken at the suggestion of Dr. Albert Tyler, who had calculated from published figures that a close relation between these two events was likely. The authors are indebted to Dr. Tyler for proposing the investigation and for critically reading the manuscript.

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[1] This investigation was supported in part by the Research Fund 171 of the University of Washington. A report of these studies was given at the meeting of the Western Society of Naturalists, December, 1951.

MATERIALS AND METHODS

The eggs of *Strongylocentrotus purpuratus* (Stimpson), collected at San Juan Island, Washington, were used in this study. For each experiment the eggs of only one sea urchin were used. To induce spawning of both sexes the urchins were injected with 4.25% KCl (Tyler, 1949). The eggs were washed with filtered sea water at least three times or until 95 to 100% fertilization of the eggs was attained. If near 100% fertilization were not possible, or if the eggs were abnormal in any respect, they were not used.

From a 5% suspension of eggs, 5- or 10-ml. aliquots were taken for egg counts and for the determination of oxygen consumption and DNA content of the unfertilized eggs. After dilution of the remaining egg suspension the eggs were fertilized, washed free of excess sperm, and the original egg concentration re-established. Aliquots of approximately 10 ml. were then taken for determinations at the two-cell stage; for all later stages 10-ml. aliquots were added to 200 ml. of filtered sea water for the first experiment and to 500 ml. for the second experiment. The embryos were cultured in a constant temperature bath at 18° C. If development did not proceed normally with the survival of more than 85% of the embryos, the results were not used.

For the determination of the rate of oxygen consumption and quantity of DNA, the embryos were harvested by centrifuging. For the swimming stages the centrifuging was done with a modified Foerst plankton centrifuge constructed so that the embryos were in contact with glass or Plexiglas only. The embryos were subjected to a maximal RCF of 150 × g for 15 to 20 minutes. They showed no visible evidence of injury following this treatment.

The number of eggs was used as a parameter for the measurement of the DNA content and the rate of oxygen consumption. Egg counts were made on an aliquot of the unfertilized eggs which was diluted until there were from 100 to 200 eggs per 10 ml. Five to 10 counts were made of 10-ml. aliquots of this dilution. The aliquots were transferred to a narrow, shallow plastic trough where the eggs settled in a row and could be counted readily at low magnification. The variation in the egg counts was less than 10%.[2] This need only be considered when comparing the first experiment with the second where different egg batches were used.

As soon as possible after harvesting, the respiration of the embryos was measured with the Warburg constant volume respirometer. The concentration of eggs used varied from 5 to 10% depending on the stage of development of the embryos. The speed of shaking used was 120 cycles per minute with an amplitude of 2.5 centimeters. These conditions of crowding and shaking were not harmful to the embryos, as determined by examination after respiration measurements. The temperature at which the measurements were made was 18 ± 0.01° C.

After the rate of respiration had been determined over a period of from ½ to 2 hours, the embryos were removed quantitatively from the Warburg flasks, centrifuged mildly, and the excess sea water removed. The packed embryos were then frozen at − 35° C. until they were analyzed for DNA content. The period of frozen storage was about two weeks.

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[2] In Experiment 1, the average number of eggs is 56,000 per ml., and the 95% confidence interval is 55,300 to 56,600. In Experiment 2, the average number of eggs is 51,800 per milliliter with a 95% confidence interval of 49,400 to 54,100.

The analyses for DNA were based on determining the amount of DNA phosphorus and were made by a combination of parts of the methods of Schmidt and Thannhauser (1945) and Schneider (1945) with the modification for the direct analysis of DNA by Schmidt, Hecht and Thannhauser (1948). The separated DNA was analyzed for total phosphorus content by the method of Berenblum and Chain (1938) following perchloric acid digestion.

Abrams (1951) reports that re-precipitation of the DNA is necessary for the removal of all of the contaminating RNA. To determine if this were necessary for the material used in the present investigation, DNA determinations were made on two aliquots of unfertilized eggs without re-precipitation of the DNA and on two aliquots with re-precipitation of the DNA two times. This is a severe test of the method because the ratio of RNA to DNA is very high in the unfertilized egg. The results are given in Table I.

Table I

The effect of re-precipitation of DNA on its quantitative determination

Unfertilized egg samples	Micrograms DNA P/10^5 eggs	Average
Washed twice	0.932 0.988	0.960
Precipitated twice	0.859 0.942	0.901

The difference in DNA phosphorus obtained by the two methods may be considered negligible. Nevertheless, in the second experiment all of the samples of DNA were re-precipitated once and washed once.

RESULTS

To determine whether a correlation exists between the rate of respiration and the content of DNA phosphorus in the normal development of the sea urchin embryo, these were measured at intervals between fertilization and the early pluteus stage. The first series, Experiment 1, was carried out in February, 1951, and the second, Experiment 2, in August, 1951. Each series consists essentially of two parallel experiments in that both the respiration and DNA phosphorus determinations were made on duplicate aliquots of eggs from the original batch with the exception of the first three DNA phosphorus determinations of Experiment 1. The results are shown in Figure 1.

The typical increase in the rate of respiration is indicated by the upper two curves. Quantitatively the uppermost of these is very similar to that published by Horowitz (1940) for this species. The curves for the amount of DNA phosphorus in the embryos parallel those of the rate of respiration except for the initial hour or two during which the latter increase rapidly and the former do not. The final plateau of the curves of Experiment 1 may be the result of starvation; the larvae do not develop further than the short-armed pluteus stage without feeding.

These data indicate that in both experiments the respiration curves parallel the corresponding curves for DNA phosphorus. To help determine whether any relationship exists between the two phenomena, correlation coefficients were calculated. The coefficient of correlation, r, for the combined data of both experiments, covering the developmental period from the 2-cell stage to the pluteus, with fourteen paired values of rates of oxygen consumption and amounts of DNA phosphorus, is 0.936. This excludes the values for the unfertilized egg, which is assumed to be very different physiologically from the developing egg. The correlation coefficient for the second experiment alone, for which the data are more complete with ten paired values, is 0.978. These correlation coefficients are much higher than necessary to be significantly different from zero at the 1% level. A scatter diagram with the regression lines for the two experiments is shown in Figure 2.

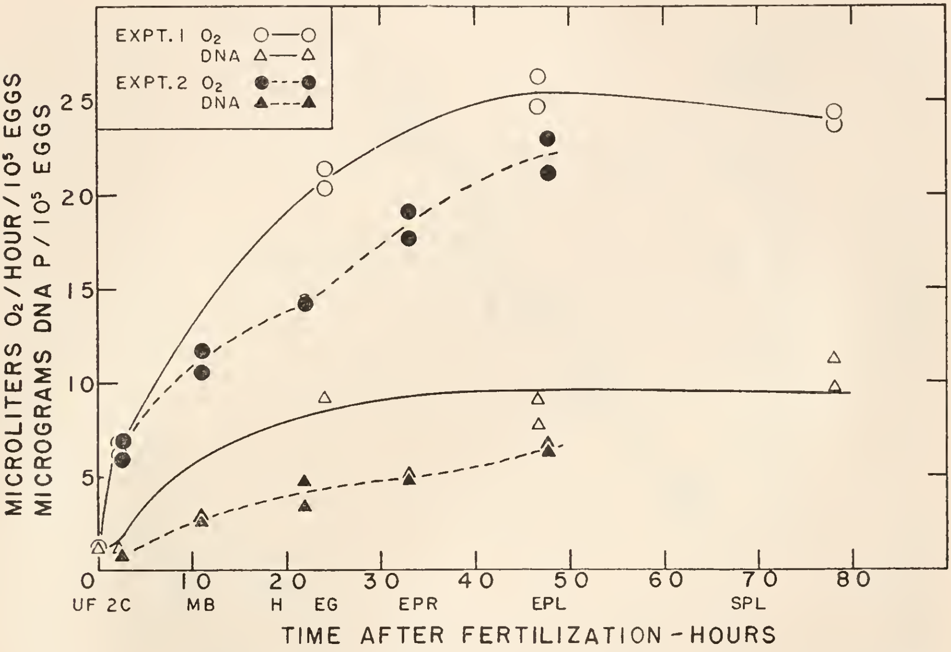

FIGURE 1. Rate of oxygen consumption and DNA phosphorus content in the embryos of *Strongylocentrotus purpuratus*. Developmental stages: UF, unfertilized; 2C, two-cell; MB, middle blastula; H, hatching; EG, early gastrula; EPR, early prism; EPL, early pluteus; SPL, short-armed pluteus.

In the unfertilized egg the rate of oxygen consumption, from the data of the first experiment, is 1.24 microliters per hour per egg while the rate of 8.34 microliters per hour per egg is the value predicted from the regression line $y = 2.04x + 6.05$ knowing that the amount of DNA phosphorus is 1.12 micrograms in the unfertilized egg. The observed value is not quite significantly different from the calculated one at the

5% level.[3] Unfortunately the amount of DNA phosphorus and the rate of oxygen consumption previous to fertilization for the eggs used in Experiment 2, for which the standard error around the regression line is much less than that for the combined experiments, were not determined so that the test for significance cannot be employed using only this second experiment. However, the data for respiration and DNA phosphorus in the unfertilized egg have been confirmed many times in other experiments by ourselves and other workers using the methods employed here, and it is quite likely that a significant difference between the respiration in the unfertilized egg and the amount expected from the regression line would be found. It may be tentatively concluded that in the unfertilized egg respiration is not related to DNA in the same manner as it is in the developing embryo.

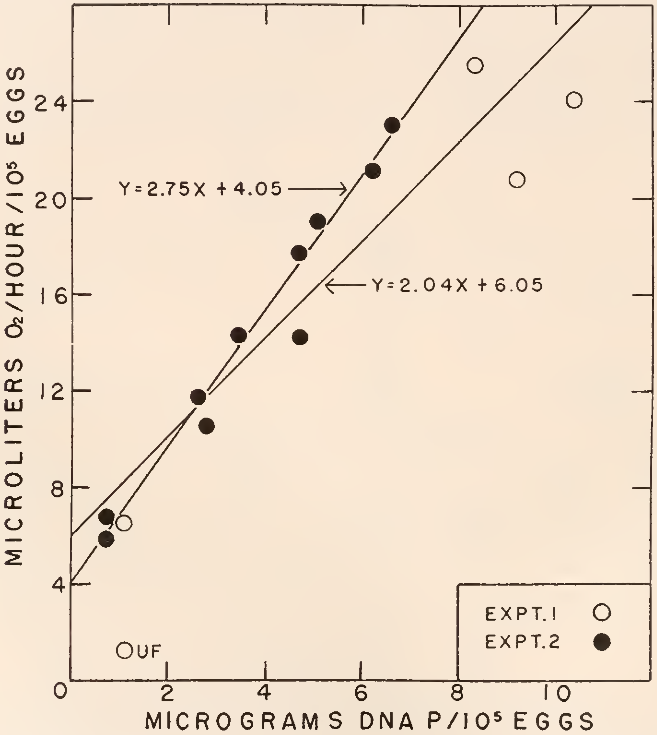

FIGURE 2. Scatter diagram with regression lines for the rate of oxygen consumption, y, and amount of DNA phosphorus, x, in developing embryos. The equation of the line for Experiment 2 is $y = 2.75x + 4.05$ and for the combined experiments is $y = 2.04x + 6.05$.

The curves obtained in Experiment 1 (Fig. 1), both for the rate of respiration and DNA synthesis, are higher than those of Experiment 2. Experiment 1 was done in February during the height of the breeding season whereas Experiment 2 was done in August, so that the dissimilarity could be due to a difference in winter and summer eggs. It was found by Fox (1938) that at a given temperature of about 20° C. for *Psammechinus* and *Paracentrotus*, material from colder waters cleaved more rapidly than material from warmer waters. In an analogous way, it may be that the rate of oxygen consumption and the synthesis of DNA would be greater for winter eggs than summer eggs when measured at the same temperature. Also, in each experiment all of the analyses were made on the eggs of one sea urchin so that the difference in the curves may be due to individual variation. The differences obtained cannot be accounted for by the variation in the egg counts.

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[3] The t test was used for this determination. A value of 2.18 for t is necessary for significance at the 5% level for 12 degrees of freedom, and the calculated value was −2.12; therefore, the probability is approximately 0.06.

Discussion

It is most probable that there is a relationship between the rate of oxygen consumption and DNA synthesis beginning with the 2-cell stage and extending into the pluteus stage. It is possible that the two phenomena are related only indirectly through other aspects of the metabolism; such indirect relationships could be very complicated and would be fruitless to consider without further information. If the relationship is direct, there are two possibilities, the first of which is that such a correlation exists because of the use of oxygen to produce energy to synthesize DNA. This relationship was indicated by Brachet (1938) on the basis of his experiments with *Chaetopterus* eggs, which were cited earlier. However, aerobic energy is used for many purposes and it is rather unlikely that a limitation of the synthesis of DNA by aerobic energy would be observable in the over-all rate of oxygen consumption. The second possibility for a direct relationship is more likely; it is that the correlation exists because of the control of the rate of oxygen consumption by the amount of DNA. The rate of respiration could be controlled by the amount of DNA if the latter were limiting the synthesis or activation of enzymes. It is known that chromosomes are largely composed of desoxyribonucleoprotein which is thought to be associated with the genetically active part of the chromosomes, the genes. Current theories of gene action propose that the primary effect of genes is to confer the final specificity on enzymes, usually one gene controlling one enzyme. However, these theories are concerned with the kind of genes and enzymes whereas the correlation discussed above is concerned with quantities. It would not be merely the number of genes present at any stage of development that dictates the activity of the respiratory enzymes, because, as was pointed out earlier, the number of cells, and, therefore, the number of genes, bears no relation to the respiration. It must be concluded, if a cause and effect relation is found to exist here, that the amount of respiratory activity is dependent on the total amount of DNA present in the embryo.

It was tentatively concluded that the respiration of the unfertilized egg is lower than expected judging from the amount of DNA phosphorus present. It is possible that before fertilization the activity of enzymes previously stored in the eggs is suppressed and respiration is low. After fertilization these enzyme systems could function at a greater capacity possibly due to a protoplasmic rearrangement (Brachet, 1950). This rearrangement is reflected in the increased rate of oxygen consumption immediately following fertilization, which, therefore, does not need to involve synthesis of DNA or new enzymes. After the utilization to full capacity of these stored enzymes, the synthesis or activation of more enzymes would be dependent on the synthesis of DNA, and from then on, the rising rate of oxygen consumption would parallel the increasing amount of DNA.

The values obtained for DNA phosphorus in this investigation approximate those obtained by Schmidt, Hecht and Thannhauser (1948) and Villee, Lowens, Gordon, Leonard and Rich (1949). Both groups of workers analyzed the eggs of *Arbacia punctulata* by the method of Schmidt and Thannhauser. Schmidt and coworkers found that the DNA phosphorus per embryo varied between 0.6 and 0.9 × 10^{-5} micrograms in the unfertilized egg and increased to 10.7 × 10^{-5} micrograms in the 24-hour pluteus. Villee and co-workers found an increase from 1.2 × 10^{-5} to 7.0 × 10^{-5} micrograms of DNA phosphorus per embryo from the third to the twentieth hour of development. The comparable results of the present study are 1.1 × 10^{-5} micrograms of DNA phosphorus per embryo in the unfertilized egg and 10.4 × 10^{-5} micrograms in the pluteus.

SUMMARY

1. Following an abrupt rise in rate of respiration of the egg of the sea urchin at the time of activation there is a period of continued increase extending into the pluteus larval stage. This is not explainable by means of the increase in cell number, cleavage rate, or nuclear volume.

2. In the developing embryo of *Strongylocentrotus purpuratus* (Stimpson) the increase in the rate of oxygen consumption has been found to have a very high positive correlation with the amount of desoxyribonucleic acid (DNA) phosphorus present in the embryo. The correlation coefficient was found to be much higher than necessary to be significantly different from zero at the 1% level. The regression coefficient for the linear regression of the rate of respiration on the amount of DNA phosphorus for the best studied case was found to be 2.75 microliters of oxygen per microgram of DNA phosphorus per hour.

3. The same correlation probably does not exist in the unfertilized egg.

4. If the relation is a direct one, the most reasonable interpretation of the correlation found is that the synthesis or activation of respiratory enzymes is under nucleic acid control.

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MOULTING AND GROWTH IN BALANUS IMPROVISUS[1]

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The Cirripedia differ from other arthropods in that the body is enclosed by calcareous plates which are not shed with the chitinous layers of the animal body and the inner lining of the mantle. As early as 1854 Darwin reported that members of the families Balanidae and Chthamalidae underwent periods of exuviation. He reports that Mr. W. Thompson of Belfast kept twenty specimens of *Balanus balanoides* alive, presumably in the laboratory, and on the twelfth day all had moulted once and one individual twice. The frequency of moulting over an extended period of time has not been determined accurately for any sessile barnacle, presumably because of the difficulties involved in maintaining them in the laboratory. Thomas (1944) reports that moults of a selected group of *Balanus perforatus* of uniform size but unknown age occurred about every fifth day but makes no reference to the period over which these observations were made. Other than these observations there has been no statement, to our knowledge, concerning the relationship between growth of shell plates and ecdyses nor is the frequency of moulting and the intermoult period, from the time of settling, known for any length of time in any barnacle.

The purpose of this investigation was to determine, therefore, a medium which would support growth of barnacles in the laboratory, to ascertain the time of moulting, period of intermoult, and the relationship of shell growth to the moulting cycle. *Balanus improvisus* Darwin, a barnacle which settles at Beaufort, N. C. during the winter and spring months, was selected for this study.

METHODS

Young *B. improvisus* were collected by suspending plastic squares 6″ × 6″ in the Beaufort Harbor for 24 hours. When brought into the laboratory most of the barnacles were in the pinhead stage but some were still cyprids. The latter were not considered a day old until the following day when metamorphosis was complete. Small pieces of plastic containing one or two pinhead barnacles were cut from the larger squares and placed in watch glasses which were in turn set, but not immersed, in finger bowls of sea water. Culture media of *Nitzschia closterium, Chlorella* sp., and *Chlamydomonas* sp. were obtained from Dr. T. Rice of the Shellfish Laboratory, U. S. Fish and Wildlife Service, Beaufort, N. C. and placed in watch glasses. After initial cultures were obtained from Dr. Rice, we maintained our own and sub-cultured regularly.

Daily examinations of the barnacles and surrounding medium were made to determine whether a moult was present. At this time the old culture medium was replaced by fresh.

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Four separate series of cultures of *B. improvisus* were maintained. The first series was started on March 30, 1952 with 56 one day-old barnacles and followed through 64 days. A second series was begun with 100 one day-old "pinheads" and maintained for 57 days. This series was supplied with a more uniform food source and was used for fixed material for another study. A third series of 10 barnacles was started and cultured in autoclaved, filtered sea water with no food source and maintained for 13 days. These were used to make a critical analysis of the time of the first moult. The fourth series was initiated with 38 barnacles and used principally as a source of fixed material for the first five moults. On day 32 of the first series and day 22 of the second all barnacles were transferred to plastic compartmented boxes to facilitate feeding and observation. These were placed under daylight fluorescent lamps and maintained under the same conditions as the cultures of algae. The average temperature of the culture media in these studies was 20° C.

Daily rostral-carinal and lateral measurements of 204 barnacles in the four series were made and areal increments were computed to the nearest 0.01 sq. mm. Each barnacle was measured with an ocular micrometer mounted in a binocular dissecting microscope. In addition, two series of 20 and 30 barnacles, collected on glass plates and suspended in the Beaufort Harbor, were measured daily for 26 and 12 days, respectively, to obtain information concerning growth under natural conditions as compared with those grown in laboratory cultures.

RESULTS AND DISCUSSION

Moulting

It has been determined from this study that *B. improvisus* undergoes frequent and regular ecdyses. The results are given in Tables I, II, III, and IV and Figures 1, 2, and 3.

Series I, consisting of 56 barnacles, passed through 20 moults in the course of 63 days. This series received only that food which was available in sea water from day 1 to 6. On day 6 they were placed in an autoclaved and filtered culture of *Nitzschia closterium*. This was changed daily for 25 days, at which time a mixed culture of *Chlamydomonas* sp. and *Nitzschia closterium* was used. An examination of Figure 1 and Table I indicates that *B. improvisus* will complete the first few moults even though an adequate food source is not available. This would seem to imply that the animal possesses stored food which is carried over from the cyprid to the pinhead, as reported by Doochin (1951). Thereafter an adequate source of food must be available or the barnacles will die. The first nine moults are confined to a more limited span than those from moult 10–20 (Fig. 1). However, if each individual on the curve is considered there is uniformity in the intermoult period. The average intermoult period for 40 barnacles of this series is shown in Table II. The variation in time exhibited by certain individual barnacles must be due to something other than food source since this was the same for each barnacle, even though cultured separately.

The second series of 100 barnacles was fed a mixed culture of *Nitzschia closterium* and *Chlamydomonas* sp. from day 1 through day 57. After 57 days most of this series was fixed, but a few individuals were maintained on *Chlamydomonas* sp. alone from day 57 to day 120, and records kept of the moulting frequency. Examination of Table III and Figure 2 shows that the moulting frequency is more uniform from the beginning and does not exhibit a pronounced lag period until after the 13th moult. Also, the intermoult periods remained relatively uniform even through the 42nd moult, covering a period of 120 days. Otherwise, the results are similar to those of the first series.

Prior to the determination of frequency of ecdysis the second moult had been considered the first. After it was determined that moulting occurred on the average of every 2–3 days, the possibility of an earlier moult was suggested which might have been overlooked. Therefore, a third series of 10 barnacles was cultured on filtered, autoclaved sea water with no food source, to make a critical analysis of the time of the first moult. Since it had been determined earlier that food was not necessary for the first few periods of ecdyses it was omitted so that a moult, if present, could be detected more readily. To insure as much uniformity of age as possible newly attached cyprids were collected and the time of metamorphosis was noted. Two

TABLE I

Moulting periods and percentage of moults in Balanus improvisus
*Series I**

Day	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
Moult 2	4 8	24 48	9 18	8 16	4 8	1 2									
3			1 2	5 10	16 32	11 22	8 16	7 14	2 4						
4						2 4	7 14	20 40	9 18	8 16	3 6	1 2			
5									7 14	19 38	13 26	7 14	1 2	2 4	1 2
Day	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28
Moult 6	2 4	6 12	16 32	11 22	6 12	5 10	3 6	1 2							
7			2 4.17	2 4.17	14 29.4	12 4.25	9 18.7	6 12.5	3 6.26						
8					1 2.23	3 6.68	7 15.5	15 34.4	9 20.0	7 15.5		2 4.45	1 2.23		
9								3 6.8	7 15.9	19 34.1	9 20.4	6 13.6	2 4.55	1 2.28	2 4.5
Day	23	24	25	26	27	28	29	30	31	32	33	34			
Moult 10	3 7.5	3 7.5	10 25	11 27.5	5 12.4	5 12.4		1 2.5	2 5						
11			3 7.5	1 2.5	8 20	12 30	6 15	3 7.5	1 2.5	4 10	1 2.5	1 2.5			

First line represents the number of moults for each day.
Second line represents the per cent of moults for each day.
* Day 1–6, sea water only; day 6–33, *Nitzschia;* day 34–46, *Nitzschia-Chlamydomonas.*

TABLE I—*Continued*

Day	27	28	29	30	31	32	33	34	35	36	37	38	39
Moult	3		3	9	8	6	4	2	4	1			
12	7.5		7.5	22.5	20	15	10	5	10	2.5			
			1	2	1	3	7	12	6	4	4		
13			2.5	5	2.5	7.5	17.4	30	15	10	10		
					1		1	6	9	9	9	3	3
14					2.5		2.5	14	22	22	22	7.5	7.5
Day	33	34	35	36	37	38	39	40	41	42	43	44	45
Moult	1		2	6	6	12	7	4	1	1			
15	2.5		5	15	15	30	15.5	10	2.5	2.5			
			1		4	3	7	9	8	5	2	1	
16			2.5		10	7.5	15.5	22.5	20	12.5	5	2.5	
Day	38	39	40	41	42	43	44	45	46	47	48	49	50
Moult	1	2	3	7	7	9	5	1	1	1	2	1	
17	2.5	5	7.5	17.5	17.5	22.5	12.5	2.5	2.5	2.5	5	2.5	
Day	40	41	42	43	44	45	46	47	48	49	50	51	52
Moult	1	1	1	7	1	2	6	5	14	3	1	1	2
18	2.5	2.5	2.5	17.5	2.5	5	15	12.5	22.5	7.5	2.5	2.5	5
Day	43	44	45	46	47	48	49	50	51	52	53	54	55
Moult	1	2			4	6	7	4	6	4	3	1	2
19	2.5	5			10	15	17.5	10	15	10	7.5	2.5	5
				1			2	6	4	11	4	1	2
20				2.5			5	15	10	27.5	10	2.5	5
Day	56	57	58	59	60	61	62	63	64				
Moult	3	3	2				1						
20	7.5	7.5	5				2.5						

TABLE II

Average intermoult periods of 40 Balanus improvisus
Series I

Intermoults	2–3	3–4	4–5	5–6	6–7	7–8	8–9	9–10	10–11	11–12
Days	3.23	2.6	2.15	2.3	2.25	2.23	2.18	2.4	2.48	2.78

Intermoults	12–13	13–14	14–15	15–16	16–17	17–18	18–19	19–20	20–21
Days	2.58	1.95	1.98	2.13	2.73	3.63	3.35	3.18	3.62

days after metamorphosis 9 out of the 10 barnacles passed through the first moult, whereas the tenth died in the process of moulting. The moults were observed through a compound microscope (110 ×) so that proper identification could be made. It is now believed that barnacles of all the other series moulted on the second day, but because of the small size of the exuviae and the masking effect of the particulate food matter, they were not detected.

The fourth series of 38 barnacles was maintained on *Chlamydomonas* sp. from day 1 and used principally as a source of histological material for another study. The moulting periods and percentage of moults of this series are shown in Table IV

TABLE III

Moulting periods and percentage of moults in Balanus improvisus
*Series II**

Day	4	5	6	7	8	9	10	11	12	13	14	15	16	17
Moult	1	44	44	7			1							
2	1	45	45	7			1							
				10	35	48	5							
3				10	36	49	5							
						3	24	48	19	1	1			
4						3	25	49	20	1	1			
								4	26	42	21	3		
5								4	27	43	22	3		
										4	30	39	21	1
6										4	31	40	22	1

Day	15	16	17	18	19	20	21	22	23	24	25	26	27
Moult	6	29	35	17	2								
7	7	32	39	19	2								
			5	29	25	15	9		2				
8			6	34	29	17	10		2				
					5	21	26	16	11	5	1		
9					6	24	31	18	13	6	1		
							5	14	35	19	6	2	
10							6	17	43	23	8	2	

Day	23	24	25	26	27	28	29	30	31	32	33	34	35
Moult	11	19	35	7	3	1	2						
11	14	24	45	9	4	1	2						
		2	15	30	19	6	2	2	1				
12		3	19	38	24	8	3	3	1				
				5	15	28	16	1	3	2		2	
13				7	21	39	22	1	4	3		3	

First line represents the number of moults each day.
Second line represents the per cent of moults for each day.
* Fed a mixed culture of *Nitzschia* and *Chlamydomonas* from day 1–57.

TABLE III—*Continued*

Day	27	28	29	30	31	32	33	34	35	36	37	40		
Moult 14	1 1.5	6 9	8 12	23 34	18 27	5 7	1 1.5	1 1.5	1 1.5	1 1.5	1 1.5	1 1.5		
Day	28	29	30	31	32	33	34	35	36	37	38	40	43	
Moult 15	1 1.5	2 3	4 6	6 9	19 29	11 17	8 12	4 6	1 1.5	3 4.6	3 4.6	2 3	1 1.5	
Day	30	32	33	34	35	36	37	38	39	40	41	42	44	45
Moult 16	2 3	6 9	2 3	5 8	6 9	5 8	15 23	10 15	3 4.6	3 4.6	1 1.5	5 8	1 1.5	1 1.5
Day	32	33	34	35	36	37	38	39	40	41	42	43	44	
Moult 17	2 3		3 4.6	2 3	2 3	2 3	7 11	7 11	11 17	8 12	7 11	5 8	3 4.6	
18				1 1.5		2 3	2 3	3 4.6	2 3	10 17	9 15	11 20	3 4.6	
19								2 4	1 2	3 6	7 14	7 14	5 10	
20										1 3	2 5	4 11	1 3	
21													3 13	
Day	45	46	47	48	49	50	51	52	53	54	55	56	57	
Moult 17	2 3	1 2	1 2	1 2										
18	6 10	5 8	2 3	2 3			1 1.5							
19	5 10	4 8	7 14	2 4	2 4		1 2		2 4	1 2	1 2			
20	7 18	9 24	2 5	1 3	1 3	1 3	2 5	1 3	1 3		1 3	2 5	1 3	
21	4 17		4 17	4 17	1 4		1 4	1 4	1 4	2 8	1 4	1 4		

and Figure 3. The sequence of moulting and the uniformity of intermoult periods appear to be related to the availability of food. From an examination of Figures 1, 2, and 3 it can be seen that barnacles maintained on a diet of *Chlamydomonas* sp. showed more uniformity than those maintained on a mixed diet of *Nitzschia closterium* and *Chlamydomonas* sp. It is our belief that this is associated with the motility of *Chlamydomonas* sp., making it more readily available to the barnacles, whereas the diatom *Nitzschia closterium* tended to clump and settle to the bottom becoming less readily available even though the culture medium was changed daily.

Kruger (1940) reported that in the stalked barnacles a new ring is added to the terga and scuta at each moult since the opercular membrane is shed with the exuvae of the body proper. An exception to this is found in members of the family Coronulinae in which the old opercular membrane is retained. The possibility of addition of new rings to the opercular plates at the time of moulting in *B. improvisus* was, therefore, investigated. We can concur that the chitinous ring is shed but have found no evidence that there is any correlation between ring number and the number of moults. In *B. improvisus,* reared from day of settling through the 42nd moult, the number of rings never corresponded to the number of moults.

TABLE IV

Moulting periods and percentage of moults in Balanus improvisus
*Series IV**

Day	2	3	4	5	6	7	8	9	10	11	12	13
Moult 1	4											
2			25	8								
			78	22								
3				4	7	15	2					
				14	25	54	7					
4					1	5	11	3	1	2		
					4	22	48	13	4	8		
5							1	2	6	2	1	
							7	14	43	14	7	
6									1	1	3	1
									16	16	50	16

First line represents the number of moults each day.
Second line represents the per cent of moults for each day.
* Fed on *Chlamydomonas* from day 1–15.

Growth

Numerous studies of the growth of various species of barnacles have been made in different parts of the world. However, the literature contains no reference to growth rates of barnacles raised on uni-algal cultures in the laboratory which will now be considered.

From the daily measurements of rostral-carinal and lateral diameters the daily increase in area of basis was computed. From an examination of Figure 5 one may conclude that barnacles fed on *Chlamydomonas* sp. from day 1 immediately show a more rapid growth than those fed on a mixed culture of *Nitzschia closterium* and *Chlamydomonas* sp. This is true even though the average area of the basis of the barnacles of Series IV was smaller on day 1. It is not surprising to note that barnacles fed grew more rapidly from day 1–6 than those without nourishment other than that which was present in standing sea water. It may be noted that from day

1–13 growth is slow, from 13 to 22 days there is an increase, but from 22 days to 37 and 46 days, respectively, the accumulated increase is even greater. It appears as though growth is following an S curve.

A comparison of the average growth of 10 barnacles grown in the Beaufort Harbor with 10 grown in the laboratory on *Chlamydomonas* sp. is shown in Figure 5. The 10 in the Harbor were eliminated at twelve days by sheepshead at the end of the settling period of *B. improvisus*. In the spring of 1953, therefore, the study

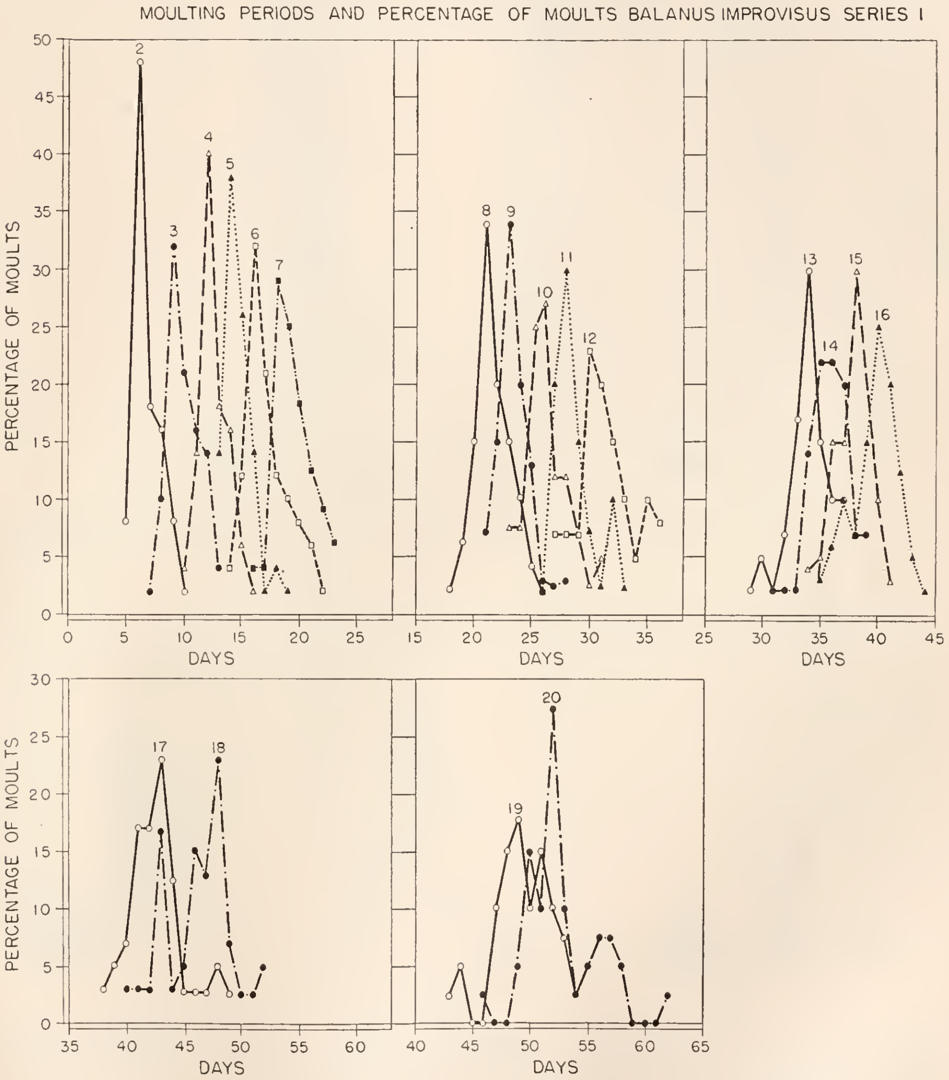

FIGURE 1. Series I received no supplemental food from day 1–6, from day 6–25 was fed *Nitzschia closterium*, from day 25–63 received a mixed culture of *Chlamydomonas* sp. and *Nitzschia closterium*. The numbers above the peaks indicate the moult number.

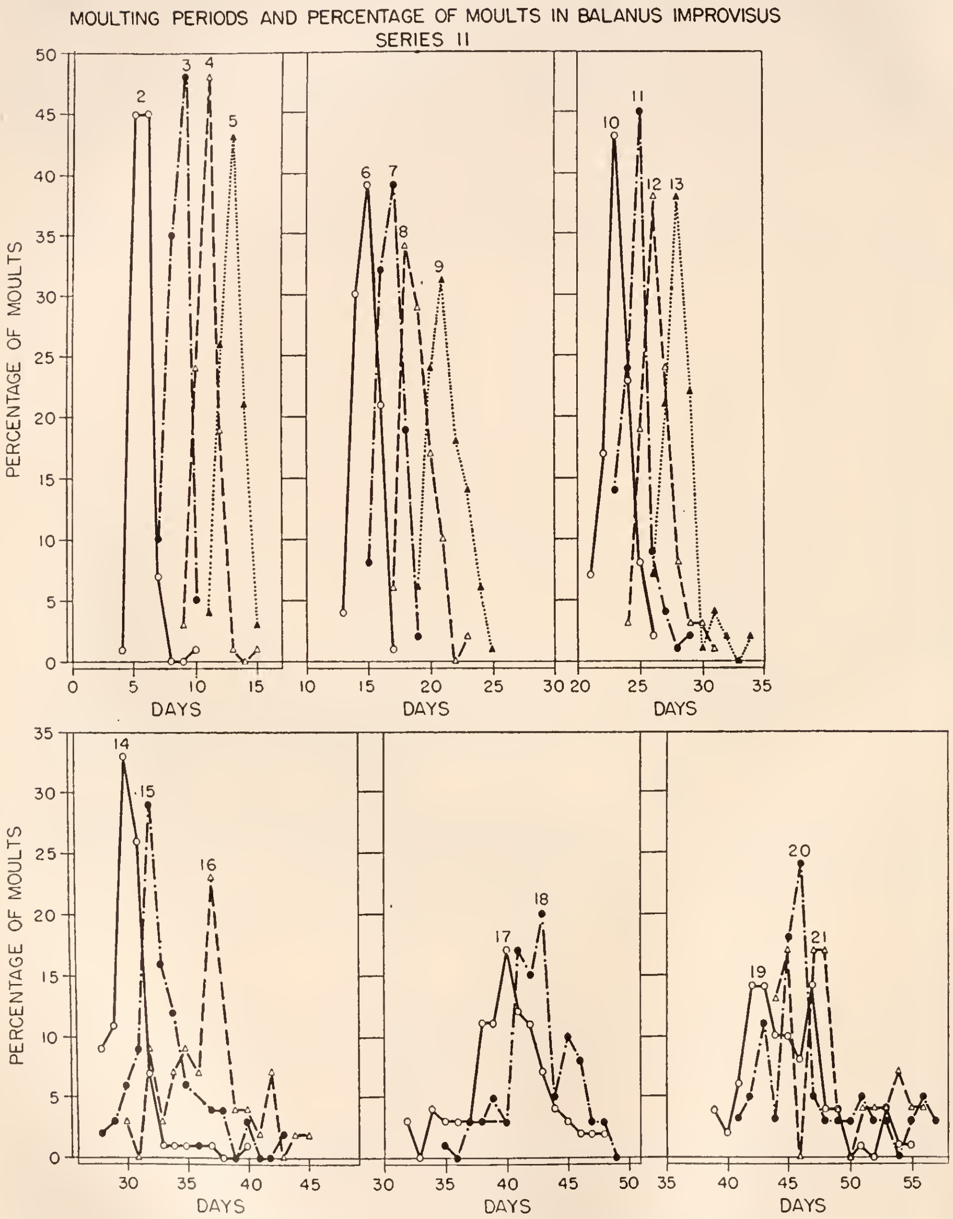

FIGURE 2. Series II received a mixed culture of *Nitzschia closterium* and *Chlamydomonas* sp. from day 1–57. The numbers above the peaks indicate the moult number.

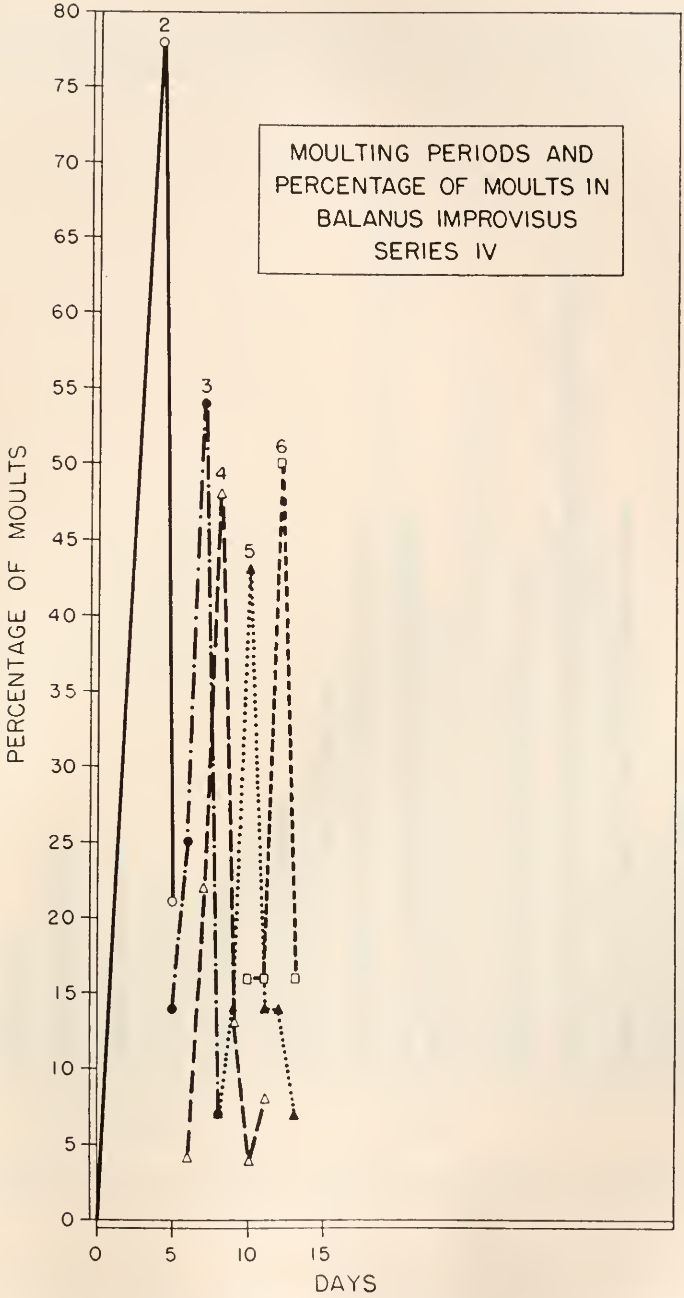

FIGURE 3. Series IV received *Chlamydomonas* sp. from day 1–13. The numbers above the peaks indicate the moult number.

of the growth of *B. improvisus* in the Harbor was repeated. The accumulative daily increase of areal growth of *B. improvisus* maintained in the Harbor for 26 days is also shown in Figure 5. Even though growth in the Harbor was greater the same characteristics are exhibited by the curve, *i.e.*, growth was slower in the beginning, increased in magnitude, and then decreased. This is even more evident if the per cent areal increase is considered.

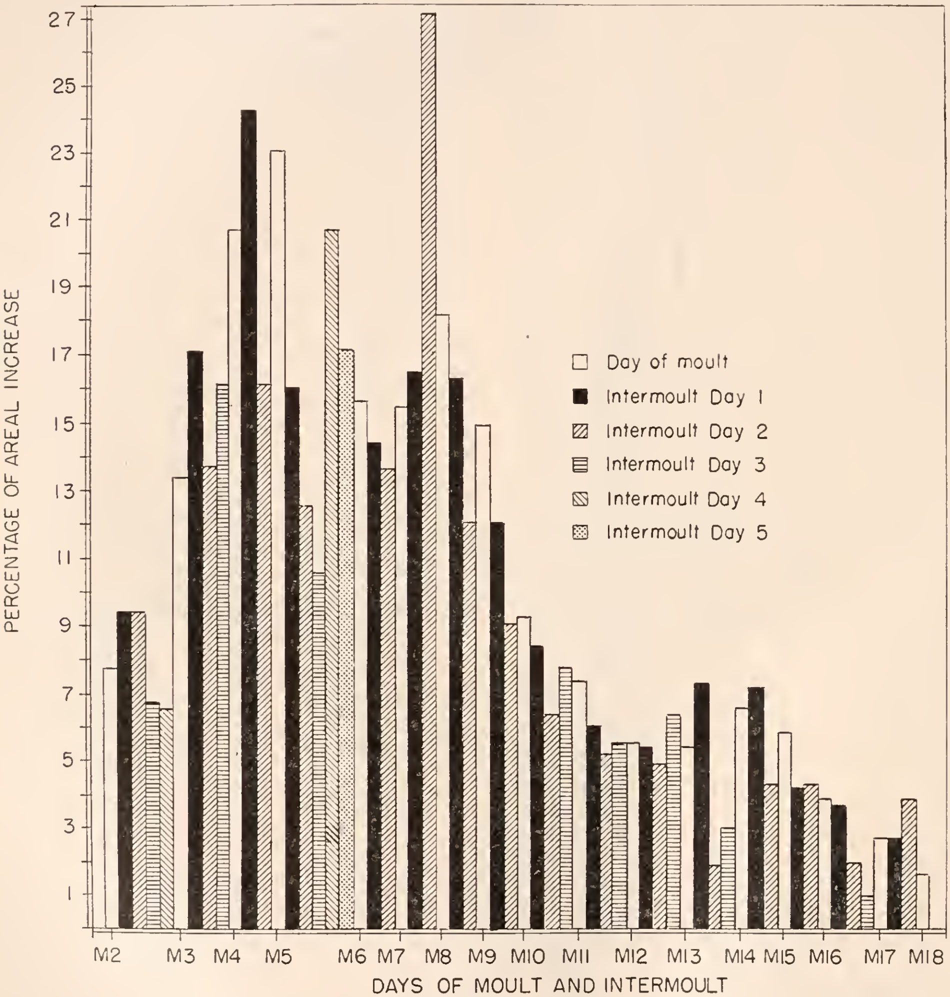

FIGURE 4. Histogram to show percentages of areal growth at day of moult and each day of intermoult in 39 *Balanus improvisus.*

An analysis of Figure 5 reveals that while the uni-algal cultures of *Nitzschia closterium* and *Chlamydomonas* sp. did not maintain a growth rate equal to that found under natural conditions where the food supply was more varied and pre-

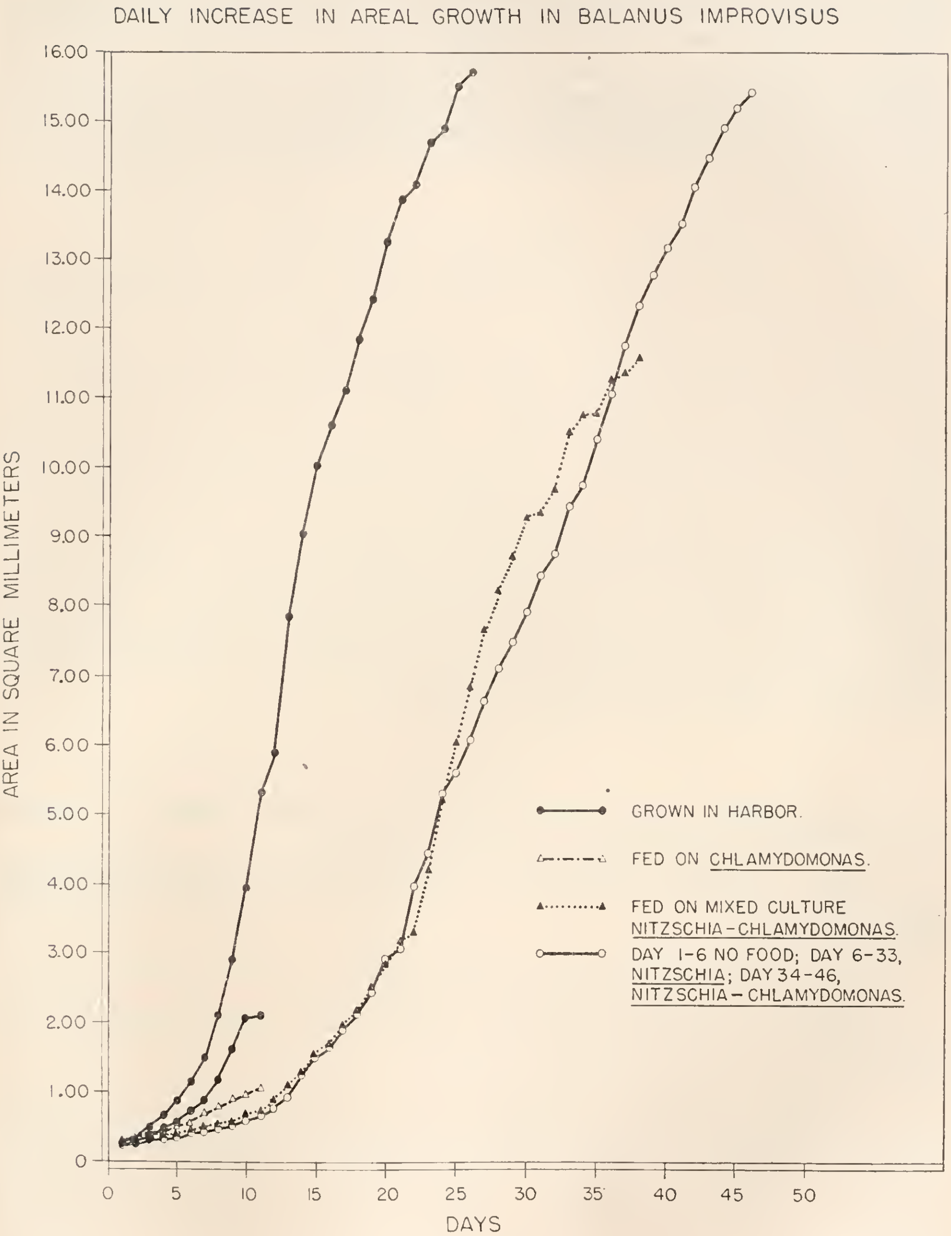

FIGURE 5. Comparison of daily increase in areal growth in *Balanus improvisus* grown in the Harbor and reared in the laboratory.

sumably of a greater magnitude, the resulting rate under artificial conditions was continuous and the food source considered adequate since the mortality under these conditions amounted to only 0.5%. The factors of social competition, crowding and predation, which appear to account for the high mortality observed in nature, were eliminated by the complete segregation of the individual barnacles in the laboratory. It is believed that the absence of water currents in the laboratory, which in the natural environment are the chief conveyers of food, would account to some extent for the lower growth rate.

Shell growth and moulting

There is no indication of any correlation between shell growth and moulting. Growth at the day of certain moults is less than days of intermoult but at other times, in the same barnacle, there is more growth at the day of moult than before or afterwards. From this type of analysis it must be concluded that growth in *B. improvisus,* although continuous, is erratic. Since individual growth rates did not show a definite trend, it was thought that an average of a group of barnacles might. The results are given in Figure 4. There is a general increase in the per cent of areal growth from the second moult through the ninth and, thereafter, there is a gradual decline. This was not only apparent in those analyzed but in all others. Nor is it necessary to have a definite amount of basis growth for a moult to occur; in fact, there are some instances in which no growth of measurable amount occurred but moulting took place. It is possible that shell height increased as well as the size of the animal body enclosed within the mural plates. A satisfactory and accurate method of measuring shell depth and body size could not be devised and thus such increases could not be included in this study.

CONCLUSIONS

1. Young *Balanus improvisus* may be reared on uni-algal cultures of *Nitzschia closterium* or *Chlamydomonas* sp. or a mixture of these.
2. *Balanus improvisus* exhibits more rapid growth in a medium containing *Chlamydomonas* sp. alone than in a mixed culture of *Nitzschia closterium* and *Chlamydomonas* sp.
3. *Balanus improvisus* grown in uni-algal cultures exhibit continuous growth but it is less than that found under natural conditions.
4. Barnacles maintained in the Harbor and in cultures show immediately after settling a slow increase in basis size, then a gradual increase which becomes more rapid, followed by a period of reduced growth.
5. The time of moulting and period of intermoult have been determined from the time of settling to 64 days for 57 barnacles in Series I, to 57 days for 100 barnacles in Series II, to 13 days for 10 barnacles in Series III, and to 13 days for 38 barnacles in Series IV, a total of 204 barnacles. A portion of Series II was followed through 120 days.
6. Moulting occurred on the average of every 2–3 days in all series maintained.
7. *Balanus improvisus* reared on *Chlamydomonas* sp. exhibit a more regular time of moulting than those grown on *Nitzschia closterium* or a combination of the two culture media.

8. Shell growth is a continuous but erratic process and there is no indication, from these studies, that there is any correlation between shell growth and moulting.

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THE ROLES OF DARKNESS AND LIGHT IN THE PHOTOPERIODIC RESPONSE OF THE TESTES OF WHITE-CROWNED SPARROWS[1]

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During the past quarter century a substantial amount of investigation has been directed towards the so-called photoperiodic response in birds, whereby it is possible to obtain an unseasonal gonadal development by increasing the length of the daily photoperiod. These investigations have been summarized very adequately by Burger (1949) and Benoit (1950). A very interesting and useful approach to an understanding of the basic mechanism involved in this phenomenon is the use of interrupted daily photoperiods. The first use of this experimental approach was apparently that of Benoit (1936). In his experiments young male ducks were maintained in dim essentially non-stimulatory light except for the experimental periods of exposure to light of stimulatory intensity when they were transferred to a light room. Benoit found that ducks exposed to 10.5 minutes of light per hour for 14 hours per night (ca. 2.5 hours total light) for a period of 16 days showed a generally greater testicular response than those which received single periods of 2.5 hours per night. Burger *et al.* (1942) obtained testicular responses in starlings (*Sturnus vulgaris*) given ten hours of light daily as a period of six hours continuous light plus eight hours of alternating 5-second light and 5-second dark periods. The entire treatment had a duration of 14 hours daily; the testicular responses were the same as those of starlings which received daily 14-hour periods of continuous light. Uninterrupted 10-hour light periods are essentially non-stimulatory in this species.

Kirkpatrick and Leopold (1952, 1953) have recently shown with bobwhite quail (*Colinus virginianus*) that, although 10 hours of continuous light daily for 37 days failed to evoke a gonadal response, the same total duration of light, but with 15–60 minutes used as an interruption of the dark period, resulted in gonadal activation. It was found that the percentage of full sexual activity after 64 days of such treatment was directly proportional to the length of the interrupting light period and inversely proportional to the length of the longer dark period. These results prompted the investigators to conclude that both the light period and the dark period are involved in sexual activation in this species, that the dark period is an inhibiting factor, and that the duration of the dark period is a major controlling factor in the response. Jenner and Engels (1952) in experiments with male slate-colored juncos (*Junco hyemalis*) and white-throated sparrows (*Zonotrichia albicollis*) have

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likewise found that it is possible to obtain testicular development with a total of 10 hours of light per day when 1¾ hours of it were used as an interruption in the middle of the dark period. Ten-hour daily photoperiods are essentially non-stimulatory in these species. These investigators have concluded that in the photoperiodic response of these species there is a (p. 354) ". . . critically important dark-dependent phase." This is somewhat difficult to rationalize in light of the testicular responses obtained by Winn (1951) and Wolfson (1952a) in the same species with exposure to continuous light.

We have recently completed a series of experiments in which we have exposed male white-crowned sparrows (*Zonotrichia leucophrys gambelii*) to several patterns of intermittent lighting. The results of these experiments are in accord with the data obtained by Kirkpatrick and Leopold (1952, 1953) and Jenner and Engels (1952); however, they lead us to feel that the assumption of an essential or active role by the dark period in the photoperiodic response is not necessary. It is the purpose of this paper to describe the results of our experiments and to present an hypothesis which rationalizes the available data on avian gonadal responses to intermittent lighting.

METHODS

Our experimental birds were captured with Japanese mist nets from the population of *Zonotrichia leucophrys gambelii* which winters in the Snake River Canyon of southeastern Washington. On February 14, after an adequate period of conditioning to cage life, five groups were placed in separate aviaries and subjected to the experimental conditions indicated in Table I. Additional wild birds (group B of Table I) were collected and examined through the course of the experiments. Each group contained a few females; because of the small numbers and the variability in responses in this sex, the data from these are not included in this report. The

TABLE I

Resumé of experimental conditions

Group	Number of males	Mean aviary temperature °C.	Daily light treatment		
			Schedules	Total hours of light	Total hours of treatment*
A	8	20	0700–1700 continuous	10	10
B**	12	6	0554–1806 continuous	12.2**	12.2**
C	7	21	0300–2100 continuous	18	18
D	10	16	0800–1630 and 2330–0100	10	17
E	15	20	Continuous 0710 to 1650 plus one minute each half-hour from 0300 to 0700 and from 1700 to 2100	10	18
F	13	21	Nine 1.11-hour periods separated by 1-hour periods of darkness (0300 to 2100)	10	18

* Time elapsed from the beginning of the first (or only) period of light to the end of the last (or only period) of light.

** Wild controls; mean daylight hours February 14 to March 16 including civil twilight.

illumination was provided by incandescent lamps at intensities of 30–40 foot candles within each cage. This is substantially in excess of the minimum required to induce a gonadal response. All lights were operated automatically. Groups A and E were kept in separate light-insulated compartments in the same constant-condition aviary. The other three experimental groups were maintained in widely separated indoor aviaries in which thermographic recording was employed to ascertain mean temperatures.

The birds in all experimental groups were kept in flight cages (12 × 24 × 18 inches), about six per cage. Food, water, and grit were provided *ad libitum*. Food consisted of red millet seed supplemented by a vitamin- and mineral-enriched poultry mash prepared by the Department of Poultry Science of the State College of Washington.

The treatments, as indicated in Table I, were continued for 29–30 days. Body weights and observations on the plumage were recorded weekly. At the conclusion of the experimental period the birds were killed and autopsied. The gonads were placed in alcohol-formaldehyde-acetic acid fixing mixture, and after several days, to allow thorough impregnation, were weighed on a Roller-Smith Precision Balance. For histologic examination, one testis from each male was imbedded in paraffin, sectioned at eight micra, and stained with acid hematoxylin and yellowish eosin.

TABLE II

Testicular weights

Group	Number	Arithmetic mean (mg.)	Range	Geometric* mean (mg.)
A	8	1.2	0.6–1.6	1.2
B	12	3.9	3.0–6.0	3.8
C	7	190	79–250	170
D	10	8.2	3.0–25	6.8
E	15	87	17–210	65
F	13	170	35–310	140

* Obtained by logarithmic transformation of testicular weights.

We are indebted to Dr. J. G. Darroch for advice concerning the statistical analyses.

RESULTS

The responses to the various treatments, in terms of testicular weights, are given in Table II. Unfortunately it was not possible to maintain the temperature of the aviary containing group D at the same level as that of the other experimental groups. Had this been possible, the response of the testes in group D would doubtless have been somewhat greater (Farner and Mewaldt, 1952) although it is highly improbable that the degrees of development of groups C, E, and F would have been approached. The test of Cochrane and Cox (Snedecor, 1946) for significance of differences between means involving samples of different sizes and differences in variance has been applied to all possible pairs of arithmetic means.

All differences are highly significant (P = 0.01) except those between the means of B–D, C–E, C–F, and E–F; however, the differences between the means of C–E and E–F are significant at the 5 per cent level. Statistical analyses of the logarithms (Bartlett, 1947) of testicular weights indicate that the differences between all possible pairs of geometric means except C–F are highly significant.

In our histologic studies we have first compared a normal series of wild and captive birds with the description of testicular development in this subspecies as presented by Blanchard and Erickson (1949). We found their description to fit our material very well, with the exception of the distinction between stages I and II (see also Blanchard, 1941) which is based on the absence of cells of Leydig in the former. We have consequently adopted Blanchard's (1941) stages in the analysis of our material although we do not separate stages I and II. The results of our histologic studies are presented in Table III. It should be noted that the 10-hour controls (group A) showed no development beyond the normal winter resting condition, whereas the wild controls, subjected to the normal spring increments in daily photoperiod, had reached stage III by March 16. In general the responses ascertained histologically closely parallel the responses indicated by testicular weights.

Table III

Testicular development

Group	Number examined	Stages* of development
A	8	8 in I–II
B	2**	2 in III**
C	7	4 in VI; 3 in VII
D	10	7 in III; 3 in IV
E	15	3 in IV; 5 in V; 5 in VI; 2 in VII
F	13	1 in V; 3 in VI; 9 in VII

* According to Blanchard (1941).

** Of the 12 birds in this group only two were examined histologically. Histologic examination of another and more extensive series of testes from birds collected in the wild reveals that all were in stage III on March 16.

Discussion

The nature of the mechanism of the photoperiodic gonadal response in birds is by no means completely understood. That the anterior pituitary gland is an essential part is patent; much of the pertinent evidence has been summarized by Burger (1949) and Benoit (1950). The ingenious experiments of Benoit (1937, 1938a, 1938b; Benoit and Kehl, 1939; Benoit and Ott, 1944) have shown further that nonocular reception of light may be involved, and that the hypothalamus is quite likely an important part of the mechanism. Wolfson (1952a) has shown that the gonadotropic effect of daily photoperiods, at least between certain minimum and maximum lengths, is a direct function of photoperiod length, and that the daily increments appear to be summated in exerting their effect. A consideration of the results of our experiments suggests the possibility that this light-stimulated mechanism is of such nature that there is involved a process which becomes active almost immediately after the beginning of the photoperiod and which has a persistent "carry-over period" of activity after the end of the photoperiod. We envision the effective duration of this "carry-over period" to be of the order of a fraction of an hour to several hours, doubtless a function of the duration and nature of the preceding photoperiod. We likewise envision its characteristics as differing in different species. Our hypothesis suggests further that the rate of testicular response would be a direct function of the summated daily gonadotropic effects of the photoperiods and the "carry-over periods." Under natural conditions, of course, there would be one of each per day. Although our data provide no direct indication of the possible physiologic basis for such a "carry-over period," several suggestions may be made in order to suggest the plausibility of the hypothesis. It is possible that the actual synthesis of gonadotropins could involve a light-dependent reaction. The "carry-over period" would then be the period of the dissipation of the gonadotropins down to an ineffective level. There is also the possibility that the actual transfer of the pituitary gonadotropins into the blood stream involves a light-dependent reaction. This is in accord with recent evidence of Benoit *et al.* (1950) which suggests that the anterior pituitary of the duck tends to release gonadotropins during light periods and to retain them during darkness. Should this be the case, the "carry-over period" might be the time required for blood gonadotropins to fall below a threshold concentration. There is further the possibility that the light-dependent reaction may be in the hypothalamus where it produces a substance which exerts a humoral control over the anterior pituitary.

With respect to the results of our experiments, this hypothesis can account for the difference between groups A and D as the effect of the additional "carry-over period" in D. The treatment and responses in group D were quite similar to those described by Jenner and Engels (1952) for white-throated sparrows and slate-colored juncos. The differences between D and E could be attributed to a longer period of gonadotropic effect in the latter because of the 17 additional "carry-over periods." The differences between E and F could be the result of the greater activity of a "carry-over period" which follows a one-hour photoperiod than of one which follows a one-minute photoperiod. Further investigations are needed to ascertain more precisely the functional relationship between the activity of a "carry-over period" and the duration of the preceding photoperiod. The data of Kirkpatrick and Leopold (1952, 1953) actually fit this hypothesis quite nicely. The inverse relationship between the length of the longer dark period and the gonadal response might be explained as the attainment of the maximum effects of two "carry-over periods" by optimum timing of the interrupting light. The direct relationship between the length of the interrupting light period and gonadal response, as reported by Kirkpatrick and Leopold (1953), conforms with our suggestion that the activity of a "carry-over period" is some direct function of the length of the preceding photoperiod. We feel that this hypothesis also rationalizes the results obtained with interrupted photoperiod by Benoit (1936) on ducks, and quite possibly also those of Burger *et al.* (1942). In the latter case it would be necessary to assume that very short photoperiods (5 seconds) were followed by "carry-over periods" of effective gonadotropic activity in the order of at least a few seconds duration.

It appears that this hypothesis might also be applicable to the mechanism of light-stimulated increase in egg production in the domestic fowl since it has been shown by Dobie *et al.* (1946), Staffe (1950, 1951), and Weber (1951) that various patterns of interrupted light give greater responses than the same amount of light as a single photoperiod. It appears that the reasoning in our hypothesis may be quite similar to that of Staffe (1951) although he is concerned primarily with the mechanism of the stimulation of increased pituitary activity with respect to maintaining a high rate of egg production. The response of sticklebacks (*Gasterosteus aculeatus*) to intermittent light as observed by van den Eeckhoudt (1947) suggests that such a mechanism may exist in this species. On the other hand it appears unlikely that the gonadal responses obtained in photoperiodic investigations of sheep (Yeates, 1949; Hart, 1950) and ferrets or mink (Hammond, 1951, 1952; Hart, 1951) can indicate such a simple rationalization for the mammals with photoperiodically-regulated reproductive cycles. In this respect it should be noted that Hammond (1952) is of the opinion that the mechanisms are basically different in birds and mammals.

Apparently in most, if not all, avian species a refractory period develops following a cycle of gonadal development. During this refractory period an increase in daily photoperiod does not evoke a gonadal development. The available evidence (Damsté, 1947; Wolfson, 1952a, 1952b) suggests that short daily photoperiods, or long dark periods, are necessary to terminate the refractory period. However, once the refractory period has been terminated, our hypothesis suggests, apparently unlike the thinking of Kirkpatrick and Leopold (1952) and Jenner and Engels (1952), that the daily dark period makes no positive contribution to the mechanism of response, although, of course, the gonadotropic effect of a "carry-over period" may be exerted during the dark period. The differences may be largely semantic; we feel, however, that our hypothesis is basically different and represents a more logical rationalization of the available data. Our hypothesis is then consistent with the suggestion of Hammond (1953) that darkness may have no active role in avian photoperiodism.

Kirkpatrick and Leopold (1952) and Jenner and Engels (1952) have emphasized possible analogies between photoperiodism in birds and in plants on the basis of their conclusions that darkness has some essential role in avian photoperiodism and the rather extensive body of evidence (Leopold, 1951; Lang, 1952) of the existence of dark-dependent reactions important in photoperiodism in plants. We feel, however, that the greatest of caution must be exerted in any serious development of such an analogy on the basis of the presently available information on birds, since there appears to be no reason to assume that the effect of darkness in the photoperiodic mechanism is anything other than that of the cessation of a process requiring light.

The hypothesis of the "carry-over period" is proposed simply as one possible rationalization of the available information on the nature of the mechanism of photoperiodism in birds. Its extension and its ultimate acceptance will rest on the results of future investigations. In consideration of the available evidence, however, we feel that two arguments favor this hypothesis: (1)it rationalizes the available information, and (2) as we have argued in our preliminary report, it is in accord with the Law of Parsimony as the simplest apparent rationalization.

SUMMARY

1. Experiments were conducted with males of a migratory race of white-crowned sparrows to ascertain the nature of response to interrupted photoperiods of several patterns.

2. The results were as follows:

(a) Testes of birds on a 10-hour continuous daily photoperiod showed no response during the 29–30 days of the experiment.

(b) Wild controls, exposed to the naturally increasing daily photoperiods from mid-February to mid-March had just begun vernal spermatogenic development and showed slight increases in testicular weight.

(c) Exposure to continuous 18-hour daily photoperiod resulted in complete spermatogenesis, or nearly complete spermatogenesis, and a marked increase in testicular weights.

(d) Daily exposure to 10 hours total light divided into two periods, 8½ hours during the day and 1½ hours in the middle of the night, resulted in a considerably greater degree of gonadal development than the wild controls.

(e) Daily exposure to 10 hours total light, as 9 hours 40 minutes of continuous light plus one minute light per half-hour for 4 hours before and after the continuous period, resulted in a markedly greater response than in (d).

(f) A still greater response was obtained with daily exposure to 10 hours total light divided into nine 1.11-hour periods equally spaced through the course of 18 hours. Spermatogenesis was as completely developed as in (c). Statistically there is no significant difference between the response in testicular weight of this group and the group subjected to continuous 18-hour daily photoperiods.

3. These results suggest to us that the light-stimulated mechanism involves a light-dependent process which responds quickly at the beginning of a period of light and soon produces a sufficient quantity of an essential substance to allow a persistent gonadotropic effect after the cessation of light. We refer to the period of gonadotropic effect after the cessation of light as the "carry-over period." It is our opinion that the assumption of an active or essential role with respect to the dark period, is not necessary in rationalizing the available information on photoperiodism in birds.

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THE PROCESS BY WHICH THE PUPARIA OF MANY SPECIES OF FLIES BECOME FIXED TO A SUBSTRATE

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It is common knowledge among students of the biology of flies that the puparia of many species are firmly fixed to a substrate. This is easy to observe in laboratory cultures of *Drosophila.* Toward the end of the larval period the larvae leave the substrate to meander for a short period on the walls of the culture bottle. The puparia are always firmly glued to the walls of the vessel. The process by which the puparia of *Drosophila* and other flies become fixed to a substrate does not seem ever to have been described. In this paper we shall demonstrate that the puparia are fixed to a substrate by means of a sticky secretion which emanates from the salivary glands.

It is well known that the salivary glands of *Drosophila* and other flies swell towards the end of larval life, and that a semi-liquid secretion fills the lumen of the glands at that time. In *Drosophila,* this is the stage most favorable for a study of the giant chromosomes of the salivary gland cells. The histological and cytological changes in the cells at the time of this secretion have been studied extensively and the presence of this secretion has been recognized and commented upon by a number of investigators (Bodenstein, 1943, 1950; Mellanby, 1938; Painter, 1945; Hsu, 1948; Blumel and Kirby, 1948; Kodani, 1948; Lesher, 1951, 1952). Some of these authors have searched for a possible function of this secretion, but none of them has recognized, or even hinted at what we consider to be the true function, namely the fixation of the puparium.

The results of this investigation have been briefly reported (Fraenkel, 1952). In this paper the process of fixation and the changes in the salivary glands will be described, while the identity of the protein in the salivary gland and in the puparial glue will be demonstrated elsewhere (Moorefield and Fraenkel, unpublished data).

Material and Methods

Most of the observations and experiments described below were carried out on the blowfly, *Phormia regina,* and on *Drosophila melanogaster.* The process of fixation to a substrate was studied by direct observation of fully grown larvae under a binocular microscope. All dissections were made in a modified Ringer solution, consisting of H_2O, 1000 cc.; NaCl, 7.5 g.; KCl, 0.35 g.; and $CaCl_2$, 0.21 g. Larvae of *Phormia* were dissected by cutting off the last segment of the body and pushing the head inwards with the blunt end of a pin while holding the body with a pair of forceps. The larvae were thus turned inside out. The major difficulty of removing the salivary glands was loosening them from the tracheae which lie in close association with the gland. If the gland was punctured in any way, it collapsed and became opaque. No satisfactory method was found other than cautiously breaking these tracheal connections with a pin. *Drosophila* larvae were dissected by submerging in Ringer solution and cutting along the ventral surface with a small piece of a razor blade.

Salivary glands have also been observed in the living insect *in situ* by pressing larvae in water under a cover slip or slide and rolling them back and forth until the glands could be seen under a microscope.

EXPERIMENTS AND OBSERVATIONS

1. Description of the secretion and fixation process

A. Drosophila melanogaster

Towards the end of the third instar the larva leaves the food and moves along the sides of the culture tube. It moves in this manner for about 12 hours, and then the movements gradually slow down until the larva comes to rest. The head continues the brushing movements which have gone on throughout larval life but now this movement describes a semicircle around the thorax. As the body gradually contracts to the barrel shape of the puparium, this movement slows down. Motion now all but stops. The mouth hooks are withdrawn but continue to move slightly back and forth. When the pupal contraction is about completed, the mouth pulsates and a clear fluid suddenly pours from it and flows along the area of contact of the body with the surface. The emission of the fluid is accompanied by a back-and-forth or pumping movement of the mouth parts and a contraction and expansion of the body. The fluid begins to harden almost as soon as it is emitted. Once the secretion is completed, the larva becomes motionless and within 30 minutes the puparium begins to darken.

B. Phormia regina

Fully fed third instar larvae leave the meat and wander about in the dry substrate provided for them, sand or sawdust, for about three days. During this period the crop becomes emptied and very much reduced in size. Several hours before the formation of the puparium the larva becomes more and more sluggish and opaque. Finally the prepupa contracts slowly to the shape of the puparium. A description of the pupal contraction and its underlying processes has been given previously (Fraenkel and Rudall, 1940). By the time the pupal contraction is nearly completed and all forward motion has ceased, the anterior segments of the body alone are still capable of some movement. Slow rhythmic contractions then start in about the fourth externally visible segment and move backwards. The larva ex-

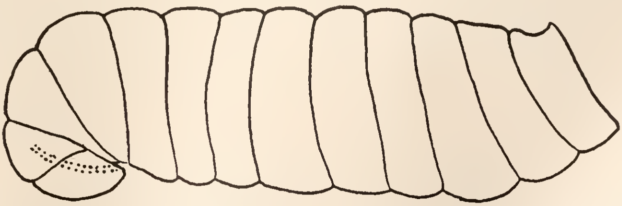

FIGURE 1. The larva of *Phormia regina* (Meig.) in the act of secreting.

trudes its proboscis, curves it toward the anterior ventral surface (Fig. 1), and then alternately inverts and extends the mouth hooks, working back and forth in motions not unlike the normal feeding motions, but directed toward its own under-surface. At the same time a pumping motion of structures within the head is visible. After the above described motions have continued for a few seconds, a clear colorless fluid emanates from the mouth and is distributed over the anterior under-surface by moving the head from side to side and back and forth in a painting or brushing movement. The fluid is tacky almost immediately after emission and hardens within a few minutes. The anterior ventral surface becomes thus firmly fixed to a surface. The time interval of actual secretion was no longer than thirty seconds.

When larvae are allowed to pupate in sand or sawdust the process of secretion is exactly the same, and in consequence clumps of sand grains or flakes of wood become firmly stuck to the anterior ventral surface. In an overcrowded culture, puparia often become stuck to each other.

Immediately after completion of this process the anterior end is permanently withdrawn into the body and the formation of the white puparium is completed. Darkening starts 15 to 30 minutes later.

2. The salivary glands of *Phormia regina*

One of the major difficulties in following salivary gland development is determination of the maturity of the larva. In this study, the size of the larva, and the amount of the contents of the crop were used as indication of development. The salivary glands of *Phormia* are similar to those described for other fly larvae. They are paired sack-like structures, elongated, extending at full size into the second abdominal segment and connected to each other at the posterior end by a small mass of fat cells.

At the time the larva leaves the food and the crop is still greatly extended with food, the salivary glands are large, distended and clear (Fig. 2a). The cells are stretched, the nucleus is distinct and the cytoplasm clear with a very fine granular consistency. The cells throughout the gland are more or less uniform in size. As the gland gets older the cells become more dense and this accounts for a milky appearance. In older glands the cytoplasm appears to shrink and a clear area is present around the cell walls.

About twenty hours after the larva leaves the food the glands begin to show a narrowing of the lumen in the posterior region (Fig. 2b). As the lumen becomes smaller, the distension of the gland in the anterior region begins to increase, the gland gets longer and begins to have a milky opacity (Fig. 2c–d). As the larva approaches the time of pupation the posterior lumen of the gland becomes more and more occluded, the cells indistinct and the fluid localized in the anterior portion, causing this area to become bulbous (Fig. 2e–f). At the moment that the larva is performing the motions as described previously and diagrammed in Figure 1, about one or two minutes before secretion, the greater part of the gland shows little if any lumen and all of the fluid is concentrated in the swollen anterior region (Fig. 2g). It is this fluid that is expelled, marking the end of larval life.

Figure 2h represents a gland immediately after secretion. The lumen has disappeared almost entirely, the cell walls have become indistinct and the whole structure is now opaque. Only a small region at the anterior end may still be full, transparent and inflated.

There are many deviations from this scheme, some of which can be explained on the basis of amount of food eaten by the larva. Underfed larvae have glands of normal length, but the lumen is small and the glands, even before the formation of the puparium, resemble somewhat those of well fed individuals after the secretion.

To determine how much the volume of secretion varies in different larvae, ten late third instar larvae were placed in each of ten petri dishes which contained fine clean sand. When all had pupated, they were collected and the amount of sand particles stuck to the surface compared. If sand particles covered more than 50% of the ventral surface or were in large clumps on the head or thorax the secretion was considered large. When only three or four sand grains were stuck to the body the secretion was considered small. The majority of larvae showed sand particles somewhat between these two. The choice is not as arbitrary as it might seem because the differences were very distinct. The variation in the amount of secretion of 100 larvae was as follows:

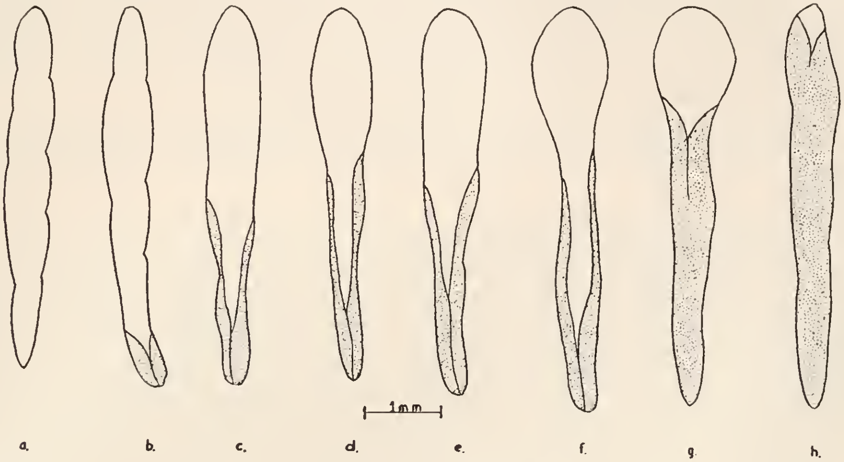

FIGURE 2. The development of the salivary gland of *Phormia regina* (Meig.) from the end of the feeding period to the formation of the white pupa. a, The gland of a 5-day old larva shortly after leaving the food. The lumen is distinct and full of fluid throughout the length of the gland. b, About 24 hours later. The lumen is beginning to disappear in the posterior region. c–d, Later in the sixth day. The closing of the lumen is more pronounced and there is a slight swelling of the anterior portion. e–f, The beginning of the seventh day, several hours before pupation. g, Immediately before secretion. h, The gland immediately after the puparium has become fixed to the substrate.

Volume of secretion	large	average	small	none
Number of larvae	29	48	17	6

The secretion of underfed larvae was usually small or absent.

In another experiment, the salivary glands both of large well fed and underfed larvae were first observed in the living larvae through the cuticle. Well fed larvae nearly always had large, clear and swollen glands, while those of underfed larvae were not distended and quite opaque. Fifty-four small larvae with empty glands and 24 large larvae with distended glands were then placed in a series of petri dishes which contained sand. Of those with unswollen glands, 10, or 19%, secreted large amounts, while 15, or 62%, of those with swollen glands secreted large amounts. However, only three of the larvae with deflated glands emitted amounts comparable to those with swollen glands.

DISCUSSION

From the observations so far reported it can only be concluded that a secretion which accumulates in the lumen of the salivary gland toward the end of the larval period is expelled immediately before the formation of the puparium is completed and that by means of this secretion the puparium becomes firmly fixed to a substrate. These conclusions are based on the following observations:

1. The secretion into the lumen of the gland develops prior to the formation of the puparium and has disappeared by the time the puparium is formed and fixed to a substrate.
2. The size of the gland as observed in the living larva *in situ* coincides with the relative volumes secreted by different larvae.
3. In a subsequent investigation (Moorefield and Fraenkel, unpublished data) a comparison was made between the total amino acid composition of the secretion from the lumen of the salivary glands and of the puparial glue collected from fully formed puparia. The same 15 amino acids were found in the proteins of both materials, and in addition glucosamine and free lysine, and the two materials proved to be identical within the limits of the paper chromatographic techniques employed.
4. Finally, if we were to argue that the prepupal secretion did not originate in the salivary glands, what other organ could be offered in their place? In fly larvae there are only two other organs large enough to harbor the amount of fluid which is ultimately discharged, the crop and the mid or hind gut. The crop becomes almost entirely reduced prior to the formation of the puparium and what little material occasionally remains is highly colored. Just before puparium formation the larva empties the remaining contents of the intestine through the anus. Thus by exclusion, we are again led to the salivary glands.

The enormous increase in size of the salivary glands during, and especially toward the end of the third larval instar has been observed by Ross (1939), Bodenstein (1943), Painter (1945), Hsu (1948) and Lesher (1951). Some of these authors ascribe this growth to the accumulation of secretory substances, which have been variously described as secretory globules (Ross, 1939; Painter, 1945) or deutoplasmic substances (Lesher, 1951). At the time of formation of the puparium these globules have largely disappeared from the cytoplasm of the cells (Painter, 1945). Bodenstein (1943) objected to an interpretation of these cellular inclusions as a true secretion, since he could not see a function for such a secretion at a time when feeding has completely ceased. Instead he claims these changes to be the result of a beginning histolysis.

Hsu (1948) and Lesher (1951) closely followed this argument. They recognized the passing out of these granules and their accumulation in the lumen of the salivary glands and ascribed it to the histolysis of the cells. Hsu interpreted the function of this material as that of food storage. Lesher (1952), on histochemical evidence, interpreted these substances as a conjugated protein of the nature of a mucopolysaccharide, with carbohydrate bonded to protein, and suggested its function to be that of a chitin precursor.

Kodani (1948), following earlier work by Blumel and Kirby (1948) recognized that the enlargement of the salivary glands was due to the appearance of material in the lumen of the gland. He collected the accumulated material, recognized its proteinous nature and identified by paper chromatography 15 amino acids. According to his description, the secretion appears in the lumen and later disappears. He finally suggested that it might form part of the pupal body or participate in chemical processes of histolysis. All these authors seem to have been confused and misled by the task to describe and interpret the appearance of what undoubtedly looked like the product of a secretory activity at a period when all feeding has ceased and no such activity was expected to occur. The ultimate function of this secretion as the glue which fixes the puparium to a substrate finally disposes of this dilemma.

All the evidence presented so far in this discussion was concerned with *Drosophila*. Comparable investigations are so far lacking for other flies, although there is little doubt that similar cycles of secretion may be assumed to exist there. Mellanby (1938) has commented on the enormous size which the salivary glands reach in the diapausing prepupae of *Lucilia sericata*. He interpreted this phenomenon as a storage of water, an assumption which is entirely devoid of evidence.

The particular mechanisms of the formation of the puparium in cyclorrhaphous flies is a phenomenon singular to this suborder of Diptera. The formation of a glue by the salivary glands, to fix the puparium to a substrate, therefore could not be expected to have a close analogy among other suborders of Diptera. In a more general sense, however, secretion of proteinous substances by salivary glands, the best known examples of which are the spinning of threads often to form a cocoon, are widely distributed phenomena in many orders of insects. Amongst Diptera, spinning occurs frequently in representatives of the suborder Nematocera. In Chironimidae, the larvae reinforce the walls of the tubes they form in mud by a secretion which emanates from the salivary glands (Pause, 1919), a fact which may account for the exceptionally large size of the salivary gland cells. The spinning habits of several representatives of mycetophilids have been well described (Medwar, 1935; Fulton, 1939, 1941). In *Ceroplatus testaceus* Dalm. spinning has been shown as the activity of well developed salivary glands (Stammer, 1932). Spinning also occurs among representatives of the family Simuliidae (Fulton, 1939).

It is not suggested by the authors that the formation of a puparial glue is the only function of the salivary glands of fly larvae. The production of digestive enzymes in the salivary glands during the feeding period has always been assumed, but has been demonstrated only in a few cases. However, some function other than that described in this paper, must be postulated for the salivary glands, especially in view of the fact that the puparia of many species of flies never are fixed to a substrate. This is the case with *Musca domestica, Calliphora erythrocephala, Sarcophaga falculata* and *S. crassipalpis*. Fixation has so far been observed by the writers in several *Drosophila* species, in *Lucilia sericata, Phormia terra-novae* and *P. regina*. It is indicated from a few preliminary observations that the salivary glands of species of flies which do not have this secretion, do not show the spectacular enlargement towards the end of larval life, as found in the other group. A comparison of the activities of salivary glands of many species of fly larvae would appear to be an interesting and necessary subject of study.

Some of the observations recorded in this paper were made by Mrs. E. Lichtwardt while working in this department. The authors are indebted to Dr. Betty Walshe, Bedford College, London and Dr. John B. Buck, National Institutes of Health, Bethesda, Md., for suggestions concerning the spinning habits of Chironomidae and Mycetophilidae.

SUMMARY

1. The puparia of many flies become fixed to a substrate by means of a secretion which accumulates in the lumen of the salivary glands toward the end of the larval period and is expelled immediately before the formation of the puparium is completed.

2. The process of fixation has been described for *Drosophila melanogaster* and *Phormia regina*.

3. The changes in the size and appearance of the salivary glands of *Phormia regina* during the accumulation of the salivary secretion and after its elimination have been described.

4. The process of secretion in the glands of *Drosophila* has been previously described by other authors, but its true function has never been recognized.

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SYNTHESIS OF PIGMENT DURING THE RECONSTITUTION OF TUBULARIA [1]

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During the reconstitution of the hydroid, *Tubularia,* a red pigment appears in the region where the new hydranth structures will arise (Loeb, 1891; Driesch, 1900; Morgan, 1901; Stevens, 1901, 1902). This pigment is presumably the carotenoid, astacene, which gives the characteristic red color to the adult hydranth and the pink color to the adult stem (Lönnberg and Hellström, 1931; Karrer and Jucker, 1950; Cohen, 1952). Little is known concerning the origin of the pigment during reconstitution, but previous work has suggested four possibilities (*cf.* Cohen, 1952).

1) The pigment could be synthesized by symbiotic red algae which are believed to occur occasionally in *Tubularia.* This is an appealing possibility because no animals are known with certainty to synthesize carotenoids (*cf.* Karrer and Jucker, 1950).

2) Pigment in the gastrovascular fluid could be taken up differentially by the reconstituting region. Algae or small crustaceans, which are regularly ingested, could provide the source of this pigment, or it could be extruded from cells located at some distance from the forming hydranth.

3) Pigment-containing cells from other regions of the stem could migrate into the hydranth rudiment. This possibility is favored by the observation (Cohen, 1952) that the coenosarc thickens in the regenerating zone and becomes thinner in adjacent regions. Tardent's (1952) finding that the interstitial cells of the ectoderm can migrate extensively also indirectly supports this alternative.

4) Finally, new pigment could be synthesized in the coenosarc, either *in situ* or throughout the stem. If the synthesis were not localized in the reconstituting area, newly formed pigment might then be redistributed according to alternatives 2 or 3.

A study of reconstitution in short segments of stems should permit a choice among the various origins of pigment outlined above. A reconstituting segment becomes completely enveloped by perisarc and does not feed. Although reconstitution will occur in segments of any desired length, the new hydranth actually develops in a region only about two millimeters long. Segments of minimal length are almost wholly involved in hydranth formation. Therefore, by using short segments, it should be possible to determine whether a synthesis of pigment occurs or whether some source of pigment extrinsic to the regenerating segment is essential.

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[1] This paper is a revision of a thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in the Department of Biology at Brown University, Providence, R. I.

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The following account presents the results of such a study in which both histological and spectrophotometric methods were utilized. The data obtained prove that new pigment is synthesized by the coenosarc of a reconstituting segment.

The author is grateful to Dr. Edgar Zwilling for suggesting this problem and especially to Dr. Mac V. Edds, Jr. under whose guidance the work was carried out. Acknowledgment is also due to Dr. Paul F. Fenton and to Dr. William Montagna for their assistance with the technical aspects of this investigation.

Materials and Methods

Colonies of *Tubularia crocea*, furnished periodically by the Supply Department of the Woods Hole Marine Biological Laboratory from August, 1952 to January, 1953, constituted the experimental material. Experiment 1 was begun at the Marine Biological Laboratory and completed at the Arnold Biological Laboratory. For all other experiments the colonies, freshly collected at Woods Hole, were placed in a thermos jug containing filtered sea water at the temperature of local waters and were transported by automobile to the Arnold Biological Laboratory. Experiments were started within six to eight hours after collection since it was found that the animals autotomized their hydranths and began to decay within 24 hours when not in running sea water.

Handling of living material

Translucent, young, and healthy stems with little or no branching were selected for uniformity of cross-sectional diameter and coloration. Stems with a caliber of from 0.5 to 1.0 mm. were cut into segments from 1.5 to 2.0 mm. in length. Six hundred to 1000 such segments were allowed to reconstitute in three or four large finger bowls, each containing about 100 cc. of filtered sea water. In Experiment 1, begun at Woods Hole, the segments reconstituted at room temperature (20–22° C.); in all subsequent experiments, the temperature was kept between 12 and 15° C.

Reconstituting segments were analyzed at each of four arbitrarily designated phases of the regenerative process. Approximately one quarter of the segments was removed from the culture bowls at each of the four phases. In the latter three phases only segments with visible indications of regeneration were selected for analysis. The segments were placed on filter paper and rolled individually to remove any pigmented algae or actinulae larvae which might be adhering to the perisarc. After a period of drying in air at room temperatures which were relatively constant during the experiments, the segments were weighed. The segments of Experiment 1 were weighed one to two hours after removal from the culture bowls; those of Experiment 2, within 20 to 40 minutes after removal. To minimize the variation in wet weight due to evaporation during shorter drying periods, the time interval from the removal of the first segment to the weighing was standardized at 35 minutes in all subsequent experiments. A period of 35 minutes was selected after a preliminary comparison of weights determined after 35 minutes of drying in air, with weights determined after over-night dehydration at 110° C., demonstrated a good correlation between them. During the last 10 minutes of the drying period, the segments were placed in the analytical balance chamber to permit the atmosphere in the chamber to come to equilibrium.

Spectrophotometric analysis

The amount of pigment in the reconstituting segments, was determined with a Coleman Junior Spectrophotometer. After weighing, the segments in a given phase of regeneration were transferred to a test tube containing 2 cc. of acetone for extraction of the pigment. The segments were ground with the flat end of a glass rod until most of the visible red color had been dissolved (ca. 5 minutes). The mixture was then stored for an extended period at 12° C. (4 to 6 weeks in Experiment 1; two days in Experiments 2–6) to extract any remaining pigment.

In Experiment 1 after acetone extraction, each acetone-extract, plus a 0.5-cc. acetone wash of the residue, was directly diluted to 5 cc. in a volumetric flask; optical densities were then determined. Since all of the optical densities obtained in this experiment were below 0.05 optical density units, a micro-method was employed in all subsequent analyses (Experiments 2–6).

In this procedure, after extraction with acetone, each acetone extract, plus a 0.5-cc. acetone wash of the residue, was transferred to another test tube and evaporated to dryness (overnight vacuum desiccation). The residue of the once-extracted segments was re-extracted for two days with 2 cc. of acetone. This supernate, plus a 0.5-cc. acetone washing of the residue, was desiccated in a similar manner. The first and second desiccated extracts were then taken up separately in acetone delivered from calibrated volumetric micro-pipettes. A 0.3889-cc. micro-pipette was used in Experiment 2; a 0.1565-cc. micro-pipette in Experiments 3–6. The pigment solutions were transferred to cork-stoppered, standardized micro-cuvettes made from No. 5 Pyrex tubing for the Coleman Junior instrument. To minimize evaporation, all dilutions were carried out in the open freezing compartment of a refrigerator, using previously chilled acetone and equipment. Optical densities were then determined.

The maximum absorption of the extracts fell between 460–470 mμ (*cf.* Cohen, 1952); their optical densities were therefore compared at 470 mμ. The absorption values of the first and second extracts were added, and their sum divided by the wet weight of the segments originally extracted. Since the dilutions employed in Experiments 1 and 2 were different from those used in Experiments 3–6, a dilution factor was introduced to make all absorptions directly comparable.

Histological methods

For histological study of pigment distribution, tissues were fixed in a solution of three parts absolute alcohol-one part acetic acid, and were then sectioned in paraffin and stained with iron hematoxylin. Pigment distribution was also examined in squash preparations of living material viewed either with polarized or white light.

The sudanophilia of adult hydranths and of several reconstituting segments was analyzed, using Baker's (1944) method with the following modification: the tissues were fixed for 20 minutes in a solution containing 10 cc. of formalin, 10 cc. of 10% calcium chloride and 80 cc. of filtered sea water. After three rinses in tap water, the pieces were imbedded in 25% gelatine and cut at 10 μ with the freezing microtome; sections were stained with Sudan black B for 8 minutes.

EXPERIMENTAL RESULTS

The phases of reconstitution selected for study are shown diagrammatically in Figures 1a–1d. The *zero point phase* (Fig. 1a) includes pieces selected immediately after the last segments had been amputated at the start of a given experiment; from 0 to 1½ hours had elapsed since the segments had been isolated and most of their cut ends had already healed. The first visible indication of reconstitution appears at the *condensation phase* (Fig. 1b) after 34–35 hours at 12–15° C. At this time, a circumferential band of pinkish-red pigment, some 0.5 to 2 mm. in length, becomes localized in the future distal half of the segment. After approximately 40–45 hours

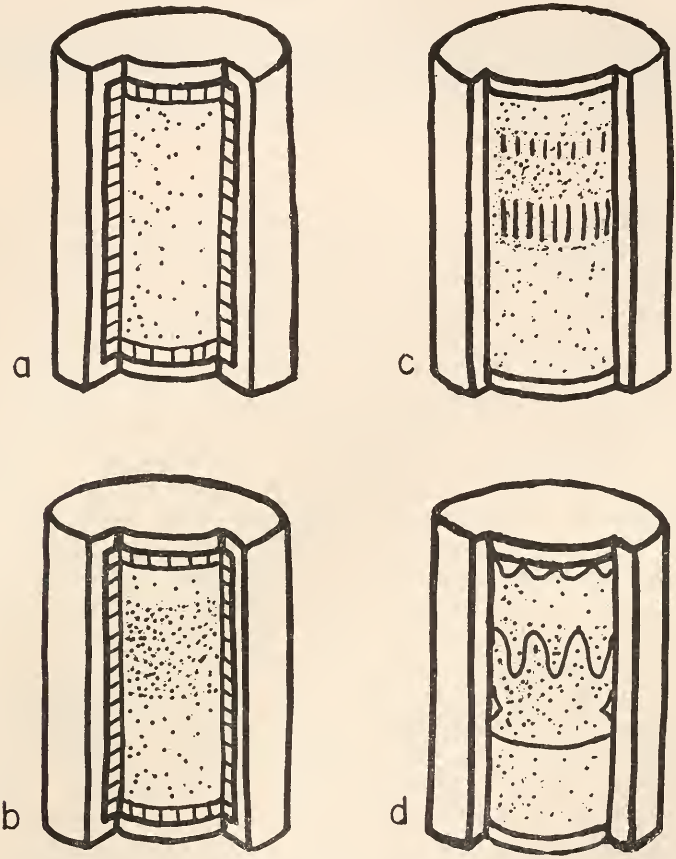

FIGURE 1. Three dimensional representation of reconstituting *Tubularia* stem segments. The characteristic red pigment distribution is shown by stippling. The clear outer covering represents the perisarc and the hatched layer in a and b, the ectoderm. a, Zero point phase segment showing pigment in the exposed endoderm. b, Condensation phase segment with band of pigment in the future hydranth endoderm. c, Striation phase; the proximal and distal pigmented striations are visible through the intact ectoderm (within the band of pigment). d, Intraperisarcal hydranth phase segment showing the unemerged hydranth within the perisarc.

of reconstitution, the *striation phase* (Fig. 1c) can be distinguished. This phase is characterized by two parallel rows of well-defined, longitudinal red striations which have developed in the pigmented band. The final, *intraperisarcal hydranth phase* (Fig. 1d), which appears after about 60–62 hours, features a constriction separating the newly formed hydranth from the coenosarc.

Origin of pigment

As pointed out in the Introduction, the work of Cohen (1952) has suggested that the externally visible reddening which occurs during reconstitution in the area of hydranth formation is due to one of four possibilities. The following account considers each of these alternatives in turn.

Role of red algae

Ceramium and other red algae which might contribute to the reddening of the hydranth-forming region, were sought in several squash preparations of living material as well as in fixed sections of 7 adult *Tubularia* and 8 segments in different phases of reconstitution. Both solitary and colonial forms of *Ceramium,* as well as *Achrochaetium* and *Rhodochorton,* have been observed in *Tubularia,* either in the endoderm or between the endoderm and the ectoderm (W.R. Taylor, personal communication). The individual algal cell is small, rectangular in shape, and contains a readily distinguishable red pigment which is not removed by ordinary histological procedures. No such cells were demonstrable in the coenosarc. Cohen (personal communication) has already observed that only occasional *Tubularia* are infested with these organisms. His results, combined with those obtained in the present study, indicate that red algae are not indispensably related to pigment formation.

Further support for this conclusion was obtained in experiments with $CuSO_4$, a compound which has been widely used as a specific poison for fresh water and marine algae (Moore and Kellerman, 1905; Domogalla, 1926; Prescott, 1938). In experiment 4, the colonies were dipped for about two minutes into a solution of 5% $CuSO_4$ in sea water immediately prior to the amputation of segments. The bottom of the bowl to which the colonies were transferred was covered with dead algae within one to two hours. Stem segments cut from these colonies nevertheless regenerated with the normal reddening of the hydranth anlagen. Identical results were obtained in Experiment 5, in which the segments were amputated 10 days after the colonies had been treated with 5% $CuSO_4$. This observation also ruled out the possibility that extra-perisarcal algal contamination plays a role in pigment formation.

Concentrating of pigment formed elsewhere

According to this view, pigment circulating in the gastro-vascular fluid is taken up by endodermal cells of the reconstituting region. This pigment would be derived either from ingested substances or from cells lying at some distance from the reconstituting region.

This alternative is ruled out by the fact that in the present experiments, reconstitution occurred in short segments which were entirely enveloped by perisarc. They were therefore completely isolated systems with respect to acquisition of new pigment from the rest of the stem, or from feeding activities.

Migration of pigmented cells

This alternative suggests that pigmented cells may migrate from a distance into the reconstituting region. Extensive cell migration cannot be critically important, however, because pigment accumulates normally during the reconstitution of short segments which are isolated from the rest of the stem.

Localized synthesis of pigment

The above observations show that the reddening in the hydranth anlagen cannot be attributed to red algae, ingested materials, or a concentrating of pigment already present. Therefore, the reddening must be due to an increase of pigment within the reconstituting segment, *i.e.*, a synthesis of pigment by the coenosarc. Since microscopic observation is not adequate to provide direct evidence on this point, the pigment content of segments at successive phases of the reconstitutive process was determined by a spectrophotometric assay.

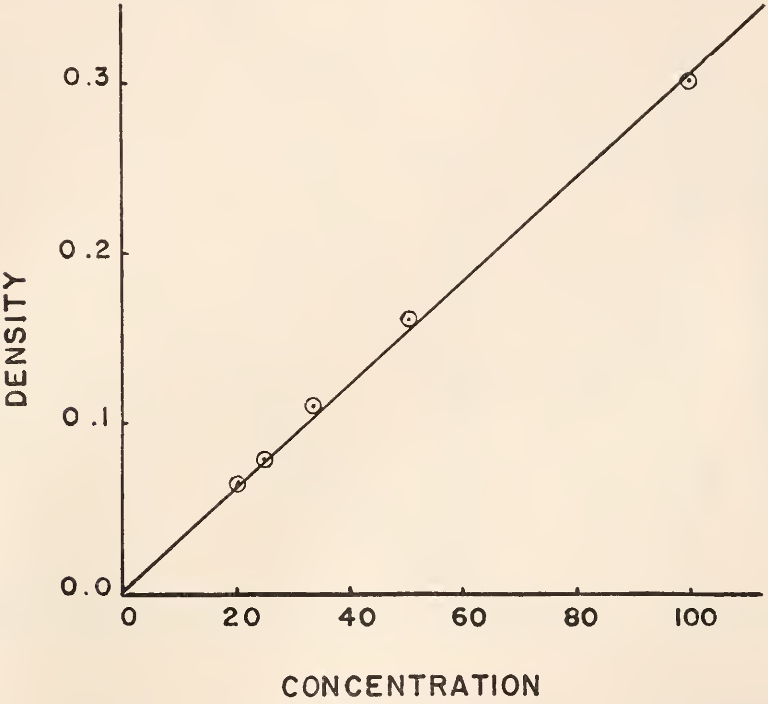

FIGURE 2. Comparison of concentration and optical density of red pigment extracts. Density is plotted along the ordinate as optical density units and concentration along the abscissa as per cent of the most concentrated.

Evaluation of method. In order to determine the validity of using optical density as a direct measure of pigment concentration, and the reliability of using the densities obtained in the lower range of the spectrophotometer, the following preliminary determination was made.

A concentrated acetone extract of adult hydranths was prepared and diluted: 1 : 2, 1 : 3, 1 : 4, and 1 : 5. The absorption values for the concentrated extract and the four dilutions were plotted against dilution expressed as per cent of the most concentrated. The resulting curve (Fig. 2) demonstrated that for optical density values of 0.05 or above, a linear relation exists between concentration and density.

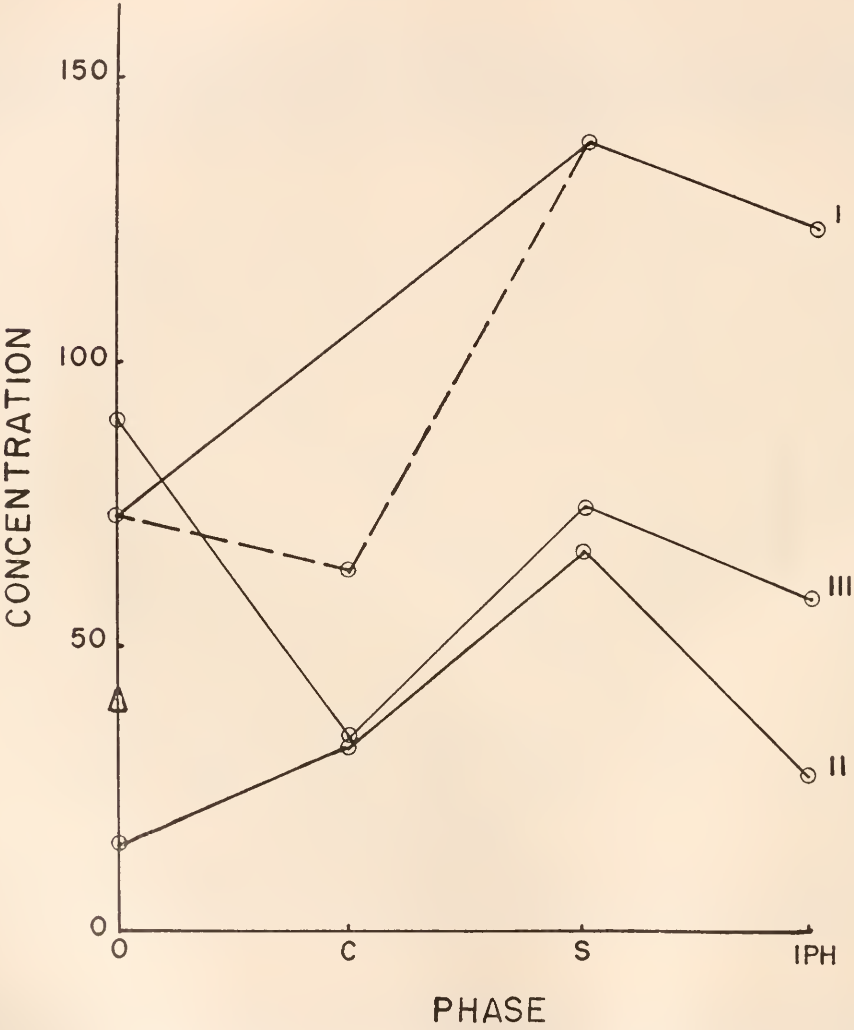

FIGURE 3. Changes in the concentration of red pigment during reconstitution. Concentration, expressed as optical density units per mg. wet weight of segments × 10^{-4}, is plotted against phase of reconstitution. O, C, S, and IPH represent the zero point, condensation, striation, and intraperisarcal hydranth phases, respectively. The optical densities were measured at 470 mμ. The triangle denotes the concentration of red pigment in segments from Experiment 6 which failed to reconstitute after four days of culturing. Curves I, II, and III represent Experiments 1, 2, and 6, respectively.

Several of the values subsequently obtained with the experimental material fell between 0.01 and 0.05 optical density units. These values appear to be trustworthy, however, since curves plotted from them were corroborated in other cases where higher values were obtained.

Spectrophotometric results. The data from Experiments 1, 2, and 6 are shown in Figure 3 (curves I, II, and III, respectively), plotted as pigment concentration per unit wet weight against phase of reconstitution. The values on which these curves are based appear in Table I.

In general, the curves show a marked increase in the amount of pigment from the zero point phase to the striation phase, followed by a moderate decrease from the striation phase to the intraperisarcal hydranth phase. Although the conditions under which the data were obtained preclude precise quantitative statements about the absolute increase in pigment, the curves do reveal the approximate range of this increase. They show: 1) that the concentration of pigment increases up to the development of the third phase of reconstitution to values some two- to four-fold above that of the base level of the zero point phase, and 2) that pigment decreases during the development of the last phase to a final value some one- to three-fold greater than that of the base level.

TABLE I

Red pigment extracts

Experiment	Stage*	Wet weight mg.	Optical density	Optical density/ mg. wet weight $\times 10^{-4}$	Optical density/ mg. wet weight at dilution of Exps. 3–6 $\times 10^{-4}$
1	O	44.4	0.012	2.3	73.2
	C	44.8	0.010	2.0	63.7
	S	74.1	0.038	4.3	137.2
	IPH	89.8	0.039	3.9	124.2
2	O	44.8	0.028	6.3	15.8
	C	45.6	0.060	13.2	33.0
	S	35.2	0.096	27.3	68.3
	IPH	36.6	0.040	10.9	27.3
6	O	66.8	0.580	86.8	86.8
	C	96.4	0.327	33.9	33.9
	S	34.0	0.252	74.1	74.1
	IPH	40.2	0.234	58.2	58.2
	N–R	33.6	0.128	41.7	41.7

* O represents the zero point phase; C, the condensation phase; S, the striation phase; IPH, the intraperisarcal hydranth phase; and N–R, the non-reconstituted phase segments in Experiment 3.

The upward displacement of curve I is probably due to the large dilution factor required to make the original optical density values directly comparable with those comprising curve III (see Materials and Methods). The first three points of curve I are interconnected with dotted lines since the data for the condensation phase in this experiment were obtained from an extract which was recovered after some spillage. The second point therefore represents at least a minimum concentration for this phase but the actual value should be higher.

The extract of the zero point phase in Experiment 6 (curve III) was observed to be redder than those of the other phases before desiccation. This indicated that the aberrantly high pigment concentration found for this phase was not due to some procedural error during dilution or spectrophotometry. The high value may be due to either: 1) a non-random selection of more highly pigmented segments to represent the zero point phase, or 2) a decrease, after the removal of zero point phase segments, in the number of pigmented larvae or algae adhering to the remaining segments. Statistically, the first alternative appears unlikely. But there is no direct evidence favoring the second and the issue must be left undecided.

Overlooking the problematical high zero point value of curve III, the graphs in Figure 3 demonstrate that the reconstituting segments gained pigment during the development of the second and third phases and lost pigment before the last phase. These findings are indirectly supported by the observation that there is a much smaller increase in pigment content in non-reconstituting segments. Regeneration of all the segments in a culture bowl never occurred. Therefore, many non-reconstituted segments were available for analysis. The pigment concentration was measured for approximately 200 segments (from the lot used for Experiment 6, curve III) which had not reconstituted after four days. The extract of these segments had an adjusted density value (triangle in Fig. 3) somewhat below that of the aberrant zero point phase of curve III but above the more reliable zero point level predicted both by curve II and the slope of curve I. On the basis of this limited evidence, it is possible that a small increase in pigment content has occurred but in any event the increase amounts to less than half of that exhibited by reconstituting segments.

Taken as a whole, the results of these experiments show that there is an increase in total pigment content of short reconstituting segments. Since the new pigment is not derived from extraneous sources, the coenosarc of the segment must synthesize pigment during the regeneration of a hydranth.

The distribution of pigment. To obtain information about the site of synthesis within the segment, the distribution of pigment in adult and reconstituting individuals was studied histologically. While these studies did not provide a definitive solution, they did reveal some pertinent facts about the form and the location in which pigment occurs. In general, two forms of pigment can be identified: 1) large ($2.6–10.3\ \mu$) granules, irregular in shape and red in color when viewed in white light, but appearing as elongate birefringent crystals when seen in polarized light, and 2) minute ($< 1\ \mu$) anisotropic crystals, white and luminescent in polarized light and dark red in white light.

Large pigment granules circulate passively in the gastro-vascular fluid of adult, living *Tubularia;* the large granules also occur intracellularly in the endoderm. In the stem, pigment granules are evident particularly in large, oval cells which are sparsely scattered through the endodermal ridges and the rest of the endoderm. Pigmented material occurs in all endodermal cells of the hypostome and is so concentrated that it is not possible to distinguish individual granules. Presumably, some of the pigment in this region is also in granular form. Since the endoderm phagocytizes pigment from the circulation (Cohen, 1952), the large intracellular granules may have this origin.

Other endodermal cells which do not contain the pigment granules, as well as the heavily colored cells of the hypostome, are filled with small, anisotropic pigment crystals. In each such cell, the crystals are homogeneously distributed and are present in large numbers. Presumably, they account for most of the gross red coloration of *Tubularia*.

Stevens (1901) also described two forms of pigment: 1) translucent, yellowish-red (1 to 8.5 μ) "plasmatic granules" which are insoluble in alcohol, and 2) small, irregular grains of red pigment which are soluble in alcohol. In some endodermal cells, Stevens found both types occurring together; in other cases, only the small grains were present. The large red granules, observed in the present investigation, appear to correspond with Stevens' "plasmatic granules"; the minute anisotropic crystals are probably identical to her irregular grains.

During reconstitution, the intracellular pigment granules appear to remain constant in number. The reddening of the hydranth anlagen is therefore probably due to an increase in concentration of the pigment crystals. It is not clear, however, whether the pigment crystals are formed *in situ* or whether there is a general synthesis in all endodermal cells of the reconstituting segment followed by a concentration of pigment or pigmented cells in the reddening area (see Introduction, alternative 4). Only further study can answer this question.

Distribution and concentration of sudanophilic substances

During the course of the experiments just described, attention was drawn to the possibility that lipid substances might be related to reconstitution. A liquid was noticed which adhered to the sides of the test tubes containing the desiccates of the first pigment extracts of Experiments 2–6. The liquid could not be evaporated by vacuum desiccation for two days. The desiccates of the zero point phases contained the largest amounts of this liquid (presumably lipid), while those of the three subsequent phases contained progressively smaller amounts. This observation prompted a histochemical analysis of the sudanophilia of reconstituting segments. The variations in the degree of sudanophilia which were detected in this analysis could not be related to the relative amounts of liquid in the desiccates. But they provided some important additional information about the reconstitution process. The methods used and the results obtained were as follows.

Studies were made on 4 to 8 sample sections (stained with Sudan black B) of three adult hydranths and stems, and of one or two segments in each phase of reconstitution from Experiments 3 and 6. The degree of sudanophilia was estimated by averaging arbitrarily assigned values for the intensity of coloration of the various regions. Five categories (0, + 1, + 2, +3, and + 4) of sudanophilia, representing a range from the least to the most intense coloration, were distinguished. In most cases it was possible to assign a value of 0, + 2, or + 4; the intermediate values of + 1 and + 3 were required only in a few instances. The average level of sudanophilia in each of the various regions of each phase was plotted against the phase of reconstitution (Fig. 4).

Adult hydranths and stems exhibit two distinct levels of sudanophilia. The endodermal cells of the hypostome are intensely colored (+ 4) while the stem endoderm contains smaller, diffusely distributed sudanophilic globules. The degree of coloration is similar (+ 2) in the endodermal ridges, in the endoderm subjacent

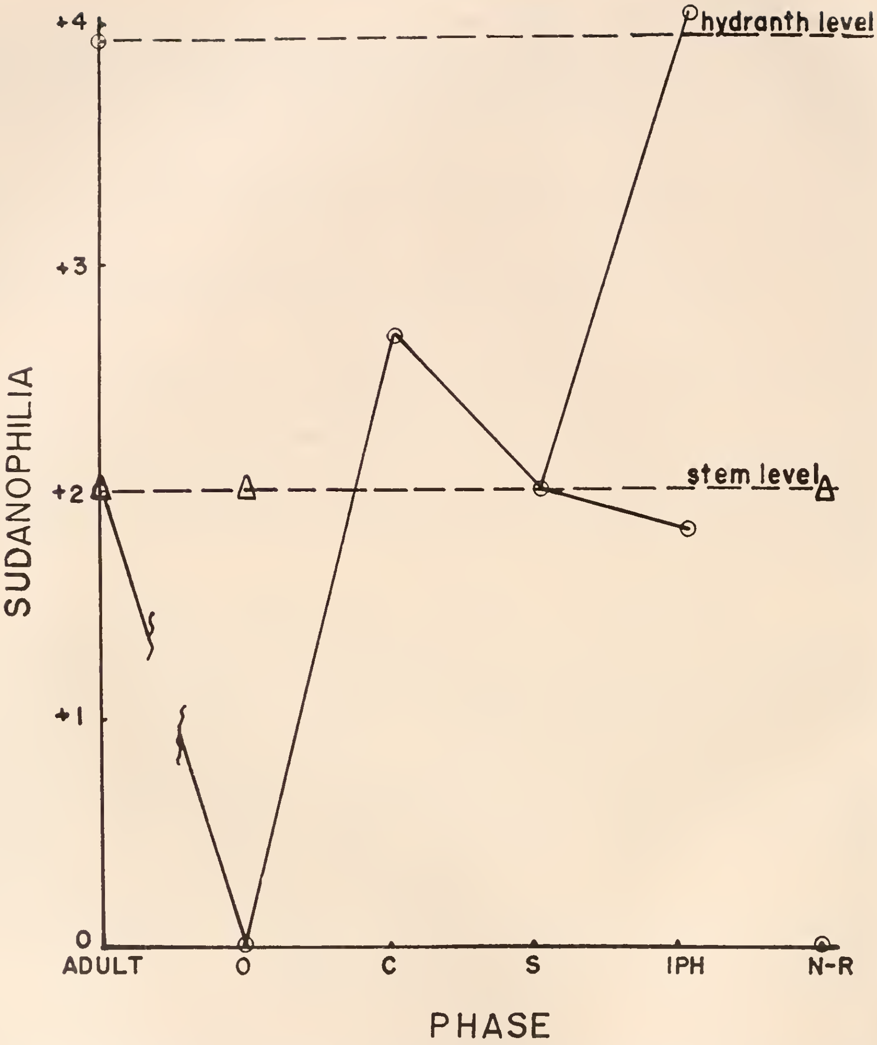

FIGURE 4. Changes in the degree of sudanophilia during reconstitution. Sudanophilia is plotted as arbitrarily designated levels of staining intensity (see text) against phase of reconstitution. N–R represents segments which had failed to reconstitute after four days of culturing; other abbreviations are as in Figure 3. Circles indicate values for the circumferential endoderm; the triangles, those for the endodermal ridges and their subjacent endoderm. The curve from the adult to the zero point phase is broken because the adults were not examined at a time in sequence with the regeneration process.

to the ridges, and in the rest of the endoderm (except for the oval cells which are sudanophobic). The pigment crystals are found in close association with some of the sudanophilic globules.

Two levels of sudanophilia also characterize the endoderm of the zero point phase segments. The cells of the endodermal ridges, which are intact only in some instances, and the subjacent endoderm, maintain a + 2 level of sudanophilia. The rest of the endoderm has dropped to a zero level. The endodermal ridges in the reconstituting end, and in some cases in both ends, break down before the appearance of the condensation phase (Morgan, 1901; Stevens, 1901, 1902); in this phase, the sudanophilia of other endodermal cells has increased to approximately a + 2 level. There is no significant subsequent change in endodermal sudanophilia up to the striation phase. Thereafter, however, a differential distribution of sudanophilic substances is evident. In the intraperisarcal hydranth phase the + 2 intensity of sudanophilia is maintained by the presumptive endoderm of the stem, whereas the hydranth anlage shows an increase to the + 4 level characteristic of the endoderm of the adult hydranth.

Segments which failed to reconstitute even after four days of culturing provide evidence that the disintegration of the endodermal ridges may be an important link in the reconstitutive process. With the exception of their endodermal ridges, which remain intact in every instance, these non-reconstituted segments are similar to the zero point segments. The sudanophilia of their ridges is + 2; that of their circumferential endoderm is 0.

Aberrant pigment extracts

Absorption spectra of all the previously described red pigment extracts (Experiments 1, 2, and 6) contained a single absorption maximum between 460–470 mμ. However, a straw-yellow solution with a single absorption maximum between 402–410 mμ, was obtained when extracts were prepared of the reconstituting segments in three other experiments (Experiments 3, 4, and 5). The procedures followed in these experiments were identical with the following important exceptions. 1) The segments of Experiment 3 were dehydrated at 110° C. overnight for dry weight determinations before extraction. 2) The colonies used for Experiment 4 were freed of algae by dipping them into a solution of 5% $CuSO_4$ in sea water immediately before segments were cut from the stems. 3) The colonies used for Experiment 5 were immersed in 5% $CuSO_4$ 10 days before segments were amputated. During the 10-day interval between treatment and amputation, the colonies were kept in filtered sea water at 12 to 15° C.; the sea water was changed every one or two days. The hydranths autotomized after the first day; reconstitution followed in the ensuing two to three days.

In these three experiments, reconstitution occurred with the normal reddening of the hydranth anlagen. The yellow extracts of Experiment 3 were obtained from segments which were heated after removal from the culture bowls. Segments from the copper-treated colonies appeared red in life but yielded yellow pigment when extracted.

The pattern of changes in the concentration of the straw-yellow pigment during reconstitution of the heat- and copper-treated segments varied from one experiment to another and no interpretation of them will be attempted. However, the possible significance of the yellow pigment will be brought out in the Discussion.

DISCUSSION

Origin of pigment. Short segments of *Tubularia* stems, which are free of red algae and isolated from extrinsic sources of pigment, develop the characteristic reddening of the hydranth-forming regions. A spectrophotometric assay has demonstrated that the total amount of pigment increases markedly in these reconstituting segments. These observations provide cogent evidence that the coenosarc of a regenerating segment synthesizes pigment. The synthesis, the nature of which is unknown, may occur throughout the whole segment or only in the hydranth-forming region.

If synthesis occurs in the entire segment, there must be a subsequent concentrating of pigment in the presumptive hydranth. The results of the present experiments offer no evidence on this point. Cohen (1952), however, has already presented evidence indicating that some re-allocation of pigment occurs during reconstitution. Using an accidentally discovered yellow form of *Tubularia,* Cohen fused 5-mm. segments of yellow stems with similar segments cut from the common red form. Subsequently, he found red pigment in the endoderm of the reconstituting "yellow" ends; these red particles gave the "yellow" hydranth a yellow-orange color. In addition, he noted that carbon particles injected into the gastrovascular fluid of a red segment were taken up differentially by the same endodermal cells which later constituted the red striations. From these observations, Cohen inferred that the linear arrangement of pigment in the primordia of the tentacles is due to a differential phagocytic activity of special endodermal cells.

Cohen's findings demonstrate that pigment already present in a stem segment can be redistributed during reconstitution. Presumably, this redistribution could occur as readily in the 2-mm. segments studied here as it did in Cohen's longer (5-mm.) segments. In a short segment, however, only a relatively small amount of pigment is present initially and synthesis of new pigment probably accounts for most of the reddening of the hydranth anlage. In long stems, containing relatively more pigment initially, re-allocation of the original stores could be a more important factor in the hydranth reddening. Therefore, it is tempting to suggest that the amount of pigment already available, either in extra- or intracellular locations, may influence the amount of pigment which will be synthesized. In this view, longer segments which contain more pigment when isolated should synthesize less pigment during reconstitution. This hypothesis could readily be tested experimentally by comparing the amount of pigment synthesized per unit wet weight of tissue in long and short reconstituting segments.

Morphogenetic significance of pigment. Previous workers have held several different opinions about the functional significance of the pigment in reconstituting *Tubularia* (see Cohen, 1952, for literature). The present work still does not permit a definitive statement but one of the following two alternatives is regarded as likely.

1) The consistent decline in the concentration of red pigment before the last phase of reconstitution (see Fig. 3), suggests that pigment or pigment-associated substances (protein, see Cohen, 1952) might be utilized in or during morphogenesis. Since the amount of tissue remained constant in the isolated segments, this decrease is a true decline in pigment concentration. There also cannot be any loss of pigment by extrusion as described by Stevens (1901, 1902) and Godlewski (1904), since the intraperisarcal hydranths were still enveloped by perisarc. Therefore, a mechanism for the breakdown of pigment may be associated with some morphogenetic activity.

2) On the other hand, the altered pigmentation—as well as the increased sudanophilia (see below)—may be merely a visible by-product of synthetic activities. If this is true, then the pigment is not causally related to morphogenesis.

Changes in sudanophilia. Reconstitution is also accompanied by marked changes in sudanophilia. Following amputation, the circumferential endoderm loses most of its sudanophilia. Concomitantly, the disintegrating endodermal ridges presumably liberate sudanophilic globules to the circulation (*cf.* the translucent globules of Stevens, 1901, 1902, and Hargitt, 1903). During reconstitution, the sudanophilia of the circumferential endoderm is gradually recovered until; at the intraperisarcal hydranth phase, levels of sudanophilia characteristic of the adult hydranth and stem are attained. In contrast, non-reconstituting segments undergo only one of these changes, namely, a decline in the sudanophilia of the circumferential endoderm.

The increase in sudanophilia of circumferential endoderm during reconstitution may result either from intracellular synthesis of new lipids, or from phagocytosis of globules previously released into the circulation—or from both. There is at present no evidence to permit a decision among these alternatives.

The relation of sudanophilia to morphogenesis is also obscure. Sudanophilic substances are increasing in the hydranth endoderm during the terminal phase of reconstitution, that is, at a time when total pigment concentration is declining. The significance of the association of these events in time is uncertain but further investigation is clearly indicated.

Disintegration of endodermal ridges. The breakdown of the endodermal ridges in reconstituting segments has been described frequently (Morgan, 1901; Stevens, 1901, 1902; Cohen, 1952). The present investigation has both confirmed these previous observations and revealed that disintegration of the ridges does not occur in segments which fail to reconstitute. These facts suggest that the breakdown of the endodermal ridges may be an important link in the reconstitutive process.

Yellow pigment extracts. The straw-yellow extracts obtained following heat or copper treatment could be due to one of the following: 1) the unmasking of a naturally occurring yellow pigment, or 2) the production of an isomeric or otherwise chemically altered form of the red pigment. There are no data which directly confirm or deny the first possibility, but evidence favoring the second has been reported by Willstaedt (1934). This investigator found that the carotenoid, astacene (presumably the red pigment in *Tubularia;* Lönnberg and Hellström, 1931 and Cohen, 1952), can be reduced by zinc dust in acetic acid-pyridine solution to give a light yellow derivative. Cohen's (1952) discovery that naturally occurring colonies of *Tubularia* may contain individuals ranging in color from red to yellow may also indicate that one type of pigment can be converted into the other.

Cohen proposes that the naturally-yellow *Tubularia* is a biochemical mutant of the red form. If this is true, and if the natural and experimentally produced yellow pigment are identical, then the heat and copper treatments have produced a phenocopy. A spectrophotometric analysis could readily decide whether or not the two yellow pigments are the same.

SUMMARY

1. The origin of the red pigment in the hydranth-forming region of reconstituting stem segments of *Tubularia* has been investigated histologically and spectrophotometrically.

2. Short (1.5 to 2 mm.), perisarc-enclosed segments, isolated from extrinsic sources of pigment (*e.g.*, ingested materials or other non-regenerating regions of the stem), reconstitute with normal reddening.

3. Histological observations have failed to demonstrate the presence of red algae within the coenosarc of *Tubularia*. Therefore, red algae do not play a critical role in the increased coloration.

4. A spectrophotometric assay of the pigment concentration in short segments at successive intervals in the reconstitutive process has provided evidence that the total amount of pigment increases during regeneration. It is concluded that the coenosarc of regenerating segments synthesizes new pigment. This finding is of interest because the pigment is presumably a carotenoid and because no conclusive evidence has previously been presented that animals synthesize carotenoids.

5. Microscopically, pigment is found in two forms: 1) large, irregular and birefringent granules sparsely distributed in the gastrovascular fluid and in certain endodermal cells, and 2) minute anisotropic crystals occurring in large numbers in the endoderm. The reddening of the hydranth anlagen is presumably due to an increase in the number of pigment crystals.

6. During reconstitution, sudanophilic substances increase markedly in the presumptive hydranth endoderm. As the distal coenosarc endoderm transforms into hydranth endoderm, it develops the characteristic adult level of sudanophilia.

7. The possible morphogenetic roles of pigment and sudanophilic materials during reconstitution are discussed. While there is no certain relation of these substances to the morphogenetic process, further analyses are suggested.

8. The breakdown of the endodermal ridges in normally reconstituting segments, and the lack of endodermal ridge breakdown in non-reconstituting segments, indicate a possible role of these structures in the regenerative process.

9. A straw-yellow pigment, chemically different from the characteristic red pigment, is obtained when reconstituting segments are treated with heat or copper sulfate. This yellow pigment may be similar to that occurring occasionally in natural populations of *Tubularia*.

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EXPLANT SYSTEMS AND THE REACTIONS OF GASTRULATING AMPHIBIANS TO METABOLIC POISONS

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Gastrulating amphibian embryos are complex systems of moving parts whose displacements relative to each other may be slowed down, or even stopped, in environments differing in but few respects from normal ones—differing, say, only in the presence of minute traces of salts that act as metabolic poisons or in being saturated with nitrogen instead of with air. But our information about such environmentally impaired systems is descriptively incomplete, even from the standpoint of gross morphology. The parts of gastrulating embryos belong to several different and embryologically interesting morphological types—to different germ layers, for example—and their relative shifts of position are of more than one morphological kind (for a recent classification see Nelsen, 1953). Yet, in general, direct observation of inhibited embryos yields only the information that they lag, morphogenetically speaking, behind embryos developing in more favorable circumstances; and it is difficult, if not impossible, to say whether a given inhibitor has affected movements of all or only of some of the germ layers, or to decide which among the distinguishable kinds of movements have been altered.

Clearly, some more adequate method of observation is needed. It is the chief aim of this paper to suggest that such a method is provided by Holtfreter's recent (1944) directions for constructing explant systems in which there occur in relative isolation more or less homologous counterparts of morphogenetic movements known since Vogt's classical work to characterize gastrulating whole embryos. Holtfreter has shown, for example, that the two parts of an explant system consisting of an endodermal ball surmounted by a piece of coated ventral ectoderm will exhibit a behavior reminiscent of epiboly. The ectoderm spreads glacier-like down over the endoderm on all sides, much as in normally gastrulating embryos. With the help of such models, observed developing in inhibitory environments, it should be possible to specify the morphological sites of action of inhibitors in as much detail as ingenuity in constructing adequate explant systems permits. A second aim of this paper is to cite the results of some exploratory experiments designed to show that information of the expected kind is in fact forthcoming. Yet we wish to stress that our relatively crude study must be refined and extended to obtain results of any genuine importance; and for this reason our discussion will be less extensive than one appropriate to more detailed experimental analysis.

METHODS

General

Suitable explants were obtained by dissecting *Rana pipiens* embryos at Stage 10 (early dorsal lip; Shumway, 1940) in full-strength Holtfreter's solution with bicarbonate at pH 8.0 (hereafter called "standard solution"). Immediately afterwards they were assembled into explant systems in inhibitor solutions (experimental systems) and in standard solution (control systems). At the same time, whole embryos were placed under experimental and control conditions exactly similar to those just mentioned. Explant systems and whole embryos were allowed to develop for 16 to 44 hours at room temperature (20 to 25° C.) except in experiments on anaerobiosis at 12 to 14° C. In the latter case, both experimental and control media were standard solution made 0.02 M in bicarbonate. Anaerobic environments were obtained and tested exactly as described by Gregg and Ornstein (1952).

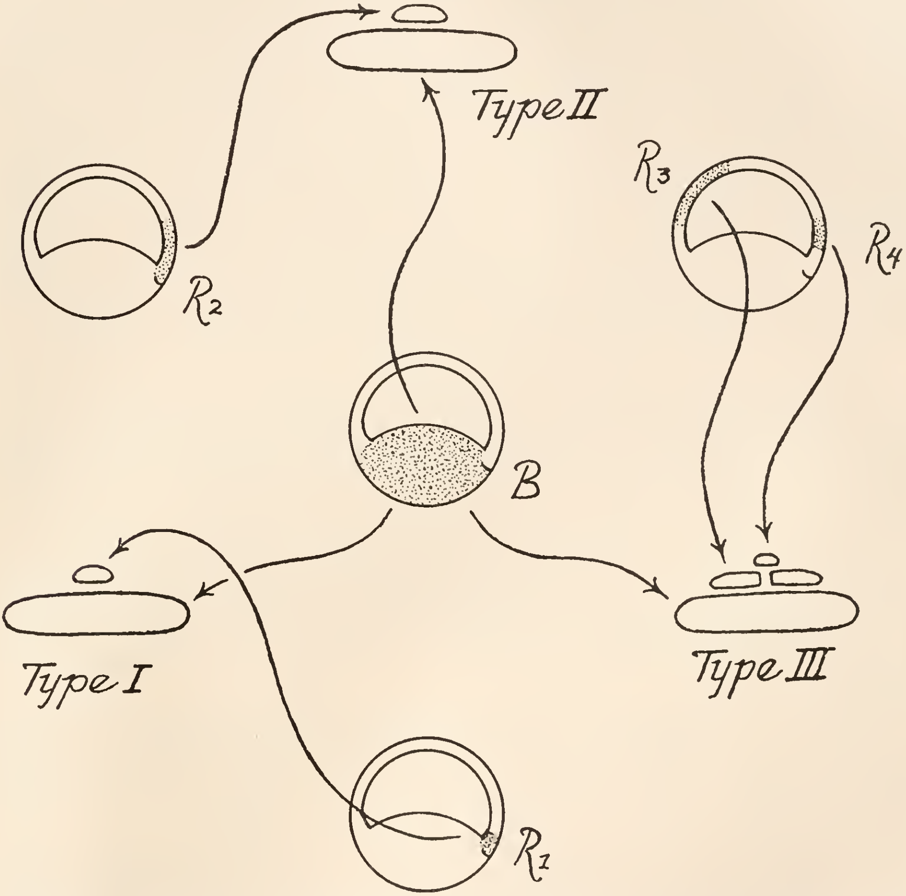

FIGURE 1. Assembly of explant systems. Whole gastrulae shown with dorsal sides to right. B: stippled area represents site of origin of endodermal bases. R_1: site of origin of Type I riders (presumptive head endoderm). R_2: site of origin of Type II riders (presumptive head endoderm and presumptive notochord). R_3 and R_4: sites of origin of Type III rider-components (presumptive epidermis and presumptive notochord, respectively). At eight, twelve and four o'clock, assembled explant systems are shown with coated surfaces of endodermal bases down, coated surfaces of riders up.

Explant systems

We have worked thus far with three different sorts of explant systems (all described by Holtfreter). Each system consists of an endodermal *base* (the entire yolk mass of a Stage 10 gastrula) and a *rider* of one kind or another (see Fig. 1).

Type I. In a system belonging to this type the rider is a small piece of coated presumptive head endoderm removed from just over the dorsal lip of a Stage 10 gastrula and set on the uncoated blastocoel floor surface of an endodermal base. During the hours succeeding their assembly, Type I systems exhibit a behavior that we call "endodermal embedding in endoderm" (briefly, "Em(en, en)"); for the riders embed themselves slowly into the bases until their surface coats are flush with the uncoated upper surfaces of the bases. (We have not observed the pitting described by Holtfreter, probably because our riders were always relatively large.) Holtfreter has studied these systems histologically (see his Fig. 16), and we have his authority for it that the riders invade their bases by the formation of "bottle cells" in a manner wholly reminiscent of a similar process presaging the first appearance of dorsal lips in whole gastrulae. We follow Holtfreter, therefore, in regarding Em(en, en) as a model of this normally-occurring morphogenetic movement.

Type II. A rider belonging to a system of the second type is a surface-coated piece of Stage 10 gastrula-tissue containing some presumptive head endoderm and also some mesoderm (presumptive notochord) set initially upon the uncoated surface of an endodermal base. Following the assembly of a Type II system, the endodermal portion of its rider executes the movement Em(en, en). The mesodermal portion of its rider may or may not invade its base, but will always elongate in the original meridional axis. We have called this behavior "mesodermal stretching on endoderm," or, for short, "St(m, en)." Holtfreter regards this movement (see his Fig. 14) as a model of convergent stretching, which is exhibited by presumptive notochord immediately prior to its invagination, or as a model of the elongation undergone by just-invaginated presumptive notochord. Again, we adopt Holtfreter's interpretation.

Type III. A system of this sort has the usual endodermal base with a rider mounted on its uncoated surface; but the rider itself is compounded of a flat sheet of coated ventral ectoderm (presumptive belly skin) pierced in the center with a hole on which is set a small piece of coated mesoderm (presumptive notochord). Type III systems, therefore, are like those illustrated in Holtfreter's Figure 22 except for the substitution of presumptive notochord for lateral marginal-zone mesoderm. Type III systems exhibit two sorts of behavior. (1) The ectodermal portion of the rider spreads out over the endodermal base in a manner that we interpret, again following Holtfreter, as a model of the epibolic extension of ectoderm in intact gastrulating embryos. We call this behavior "ectodermal spreading on endoderm," or "Sp(ec, en)." (2) The mesodermal part of the rider fuses smoothly with the ectodermal part, in an activity we have called "mesodermal fusion with ectoderm on endoderm," or "Fu(m, ec, en)." This particular behavior does not clearly model any normally-occurring gastrular event. We had hoped to be able to duplicate the events sketched in Holtfreter's Figure 22, which can be reasonably interpreted as modelling the formation of a rudimentary archenteron by mesodermal tissue, but we were never able clearly to obtain this result. Nevertheless, we report experiments with Fu(m, ec, en) because it is differentially affected (relative to other model movements) in some inhibitory environments.

Total carbohydrate

To obtain information about carbohydrate utilization by gastrula explants under anaerobiosis, dorsal and ventral explants (usually about 8 each) from Stage 10 gastrulae were divided into lateral halves. Right halves (DR, VR) were made anaerobic, left halves (DL, VL) *from the same gastrulae* were kept as controls. After 20.5–21.5 hours at 12–14° C., in standard solution made 0.02 *M* in bicarbonate, explants were homogenized in a known volume of standard solution, aliquots of brei were hydrolyzed for three hours in ca. 2 per cent HCl, proteins were precipitated by treatment with copper and acid tungstate, and reducing sugars were estimated by the method of Kirk (1950) and Stern and Kirk (1949). Other aliquots of brei were taken for dry weight measurements with a quartz helix microbalance. Under these conditions we were able to recover 98 per cent of known amounts of glucose added to brei (in the range 1–12 μg.) with a reproducibility of about 2 per cent.

The explant-types are those described by Ornstein and Gregg (1952); a fuller description of the method of weighing is to be found in this publication also.

DEVELOPMENT OF WHOLE EMBRYOS IN INHIBITORY ENVIRONMENTS

The developmental behavior of gastrulae placed at Stage 10 under inhibitory conditions is summarized in Table I. Generally speaking, we have chosen to work

TABLE I

Development of amphibian gastrulae exposed to inhibitory environments at Stage 10. Temperatures, 20–25° C.

Inhibitor	Conc'n. molar	pH	Exposure, hours	Developmental stage at end of exposure		No. clutches tested
				Exptls.	Controls*	
Azide	0.005	8.2	16–20	10^{+}–11	12–14	2
Malonate	0.045	8.3	22	11^{+}†	14	1
	0.040	8.0	17–18	11–11^{+}	12^{+}–13§	2
NaCl‡	0.045	8	18–24	11^{+}†	13–15	3
	0.022	8	18–22	12–14	13–14	2
p-chloromercuribenzoic acid	5×10^{-5}	8.2	16–20	11–11^{+}	12^{+}–15	4
2,4-dinitrophenol	1.1×10^{-4}	8.0–8.2	17–21	10^{+}	13–15	5
Sodium barbital	0.05	9.4	20	11^{+}–12^{-}	14	1
Urethane	0.1	8	44	10^{+}	18	1
	0.05	8	44	14^{+}	18**	1
95 N_2:5 CO_2	Saturated††	7.2–7.4 (calc.)	16–18	10^{+}–11^{+}	12^{+}–14	3

* In standard solution at pH 8.0.

† Head-fold induction in these gastrulae, in spite of incomplete invagination.

‡ Not regarded as a metabolic poison; see text section on malonate.

§ Another (atypical) control set developed only to Stage 11^{+}.

** Some persistent fairly large yolk plugs.

†† In standard solution made 0.02 *M* in bicarbonate.

with inhibitors in concentrations that slow or stop gastrulation-movements at the pH's indicated, and yet which result in minimum damage to exposed embryos—damage in the sense of visible cytolysis, surface-coat corrosion, and so on. Where some visible injury has been unavoidable, it will be mentioned in an appropriate place.

To reiterate a point made earlier: with some exceptions, inhibited embryos differ visibly only in minor respects from normal ones, the chief difference being that they develop more *slowly* than normal ones. In some instances, of course, developmental processes are brought to a standstill, or nearly so.

DEVELOPMENT OF EXPLANT SYSTEMS IN CONTROL AND IN INHIBITORY ENVIRONMENTS

The data will be presented piece-meal as a sequence of formulae interspersed with commentary. For this purpose a simple and brief notation has been adopted.

If R is a type of response exhibited by successful control systems, C is the set of environmental conditions regarded as relevant to R, P is the set of positive results (the movement definitely occurred) obtained in the experiments, N is the set of negative results (the movement definitely failed to occur), I is the set of indeterminate results (undecidable except by vote), and m, n, p and q are integers, then the expression:

$$\mathrm{R}:\mathrm{C};\ (m);\ n\mathrm{P};\ p\mathrm{N};\ q\mathrm{I}$$

will be read as asserting that out of m attempts to obtain responses of type R under environmental conditions of Type C, n were successful, p were unsuccessful and q were indeterminate. A fictional instance may help to make this idiom perfectly clear. The formula:

$$\mathrm{Em(en, en)}:\mathrm{NaN_3},\ 0.01\ M;\ (7);\ 4\mathrm{P};\ 1\mathrm{N};\ 2\mathrm{I}$$

tells us that out of 7 attempts to obtain endodermal embedding in endoderm in 0.01 M sodium azide, 4 were successful, 1 was unsuccessful, and 2 were indeterminate.

Parenthesized numerals preceding formulae indicate merely their order of presentation.

Control environments

Because of their almost uniformly unambiguous success, data on all the control experiments are presented together, classified according to the kind of movement studied.

(1) Em(en, en): standard solution; (24); 24P; 0N; 0I
(2) St(m, en): standard solution; (30); 29P; 0N; 1I
(3) Sp(ec, en): standard solution; (22); 22P; 0N; 0I
(4) Fu(m, ec, en): standard solution; (22); 22P; 0N; 0I

The behavior of these explant systems under control conditions (standard solution in all experiments except those on anaerobiosis; in these, standard solution made 0.02 M in bicarbonate served both as experimental and as control medium) is astonishingly reliable and reproducible. Aside from preliminary trials during which we were learning how to assemble the systems, and whose results are not reported here, we have rarely had to discard an experiment because of unsatisfactory behavior on the part of control systems. No uncontrolled experiments are reported.

Sodium azide (*NaN_3*)

The results of experiments with azide:

(5) Em(en, en) : NaN_3 0.005 *M*; (4) ; 4P; 0N; 0I
(6) St(m, en) : NaN_3, 0.005 *M*; (6) ; 0P; 6N; 0I
(7) Sp(ec, en) : NaN_3, 0.005 *M*; (5) ; 0P; 5N; 0I
(8) Fu(m, ec, en) : NaN_3, 0.005 *M*; (5) ; 5P; 0N; 0I

indicate that explant systems immersed in weak solutions of it fail to exhibit movements of the types St(m, en) and Sp(ec, en), but are perfectly normal in respect to movements of the kinds Em(m, en) and Fu(m, ec, en). Therefore, insofar as our models do not simply parody the *in situ* movements of gastrulating embryonic parts, we may reasonably suppose that whole gastrulae are blocked by immersion in azide solutions (see Table I) not because such environments are inimical to bottle-cell formation but partly, at least, because convergent stretching and epibolic spreading are somehow interfered with. Furthermore, we can say (although the morphogenetic import of this is not quite clear) that azide does not abolish whatever conditions are necessary for mesoderm and ectoderm to fuse normally.

It should be noted that in azide the ectodermal parts of riders in Type III systems fail to adhere well to the endodermal bases, and this suggests that some cell-surface feature is altered that under more favorable circumstances allows endoderm as a suitable substrate to which ectoderm can stick while spreading.

Sodium malonate (*Mal*)

Our experiments with sodium malonate have yielded results that in some respects are confusing. These are reported, nevertheless, in caution against accepting claims for specific inhibitor effects obtainable only when the inhibitors are employed in relatively high concentrations.

Reference to Table I will show that whole gastrulae are developmentally slowed in 0.045 *M* and also in 0.040 *M* malonate solutions. From a biochemical point of view, this result is rendered less interesting by the discovery, also cited in Table I, that the same effect is obtainable with 0.045 *M* NaCl. This is pointedly suggestive of a purely osmotic effect on the part of malonate. Perhaps the osmotic pressure of the blastocoel is elevated by the penetration of malonate to such an extent that the resulting increase of blastocoel hydrostatic pressure makes complete invagination impossible, or perhaps some osmotic damage to gastrular cells is incurred.

Whatever the exact situation, it is not greatly illuminated by the behavior of explant systems in malonate solution. In 0.045 *M* malonate no movements belonging to any of the four types are seen:

(9) Em(en, en) : Mal, 0.045 *M*; (2) ; 0P; 2N; 0I
(10) St(m, en) : Mal, 0.045 *M*; (2) ; 0P; 2N; 0I
(11) Sp(ec, en) : Mal, 0.045 *M*; (2) ; 0P; 2N; 0I
(12) Fu(m, ec, en) : Mal, 0.045 *M*; (2) ; 0P; 2N; 0I

Yet in 0.040 *M* malonate (in which whole gastrulae develop exactly as they do in 0.045 *M* malonate) some of the movements, notably St(m, en) and Fu(m, ec, en), occur with a relatively high frequency of success:

(13) Em(en, en) : Mal, 0.040 M; (7) ; 3P; 2N; 2I
(14) St(m, en) : Mal, 0.040 M; (8) ; 6P; 2N; 0I
(15) Sp(ec, en) : Mal, 0.040 M; (8) ; 3P; 5N; 0I
(16) Fu(m, ec, en) : Mal, 0.040 M; (8) ; 6P; 2N; 0I

The asymmetry of all these results is increased by the behavior of explant systems in 0.045 M NaCl, which, unexpectedly, behave quite normally except in respect to Sp(ec, en) :

(17) Em(en, en) : NaCl, 0.045 M; (2) ; 2P; 0N; 0I
(18) St(m, en) : NaCl, 0.045 M; (2) ; 2P; 0N; 0I
(19) Sp(ec, en) : NaCl, 0.045 M; (2) ; 0P; 1N; 1I
(20) Fu(m, ec, en) : NaCl, 0.045 M; (2) ; 2P; 0N; 0I

We have no results with explant systems in 0.04 M NaCl; but in 0.22 M NaCl they are apparently perfectly normal, and whole gastrulae are affected but little, if at all (Table I).

Incidentally, the warning with which this section opened applies to our own claim (Ornstein and Gregg, 1952) that explant respiration is reduced in 0.04 M sodium malonate. We stand by this assertion, but the experiments yielding it would now seem to need revaluating.

Para-chloromercuribenzoic acid (HgB)

This powerful -SH inhibitor is definitely capable of blocking Sp(ec, en) and probably St(m, en) ; its effect on Fu(m, ec, en) is undetermined by our experiments; and it has no effect, in the concentrations employed, upon Em(en, en) :

(21) Em(en, en) : HgB, 5×10^{-6} M; (4) ; 4P; 0N; 0I
(22) St(m, en) : HgB, 5×10^{-6} M; (4) ; 0P; 2N; 2I
(23) Sp(ec, en) : HgB, 5×10^{-6} M; (4) ; 0P; 4N; 0I
(24) Fu(m, ec, en) : HgB, 5×10^{-6} M; (4) ; 0P; 0N; 4I

It should be remarked that p-chloromercuribenzoic acid tends to thin out or corrode the surface coats of explants[1] or of whole embryos. Under the present experimental conditions there were always some loose cells lying at the outer surfaces of the explant systems and impeding observation. This accounts for our inability to decide whether Fu(m, ec, en) occurs in solutions of this inhibitor (24) and for the indeterminacy of some of the results mentioned in (22). It seems to account also for the negative results reported in (23) ; for the ectodermal portions of the riders of Type III systems, denuded by p-chloromercuribenzoate of their surface coats, behaved as though they were endodermal pieces and embedded themselves in the endodermal bases instead of spreading over them. These observations confirm Holtfreter's report (1944) that uncoated explants of almost any sort will behave in this manner, relative to an endodermal substratum.

[1] We wish to correct an inadvertent error of omission in Ornstein and Gregg, 1952: in that publication we failed to mention that explants in 10^{-4}–10^{-5} M p-chloromercuribenzoic acid become heaps of loose cells during their two-hour stay in the respirometers. Presumably this occurs because their surface coats are disrupted, for their cells are otherwise not visibly affected. This change is unaccompanied by alteration of respiratory rate, *i.e.*, the latter stays constant during the period of measurement. For weighing, the dispersed cells were removed with a braking pipette.

It is interesting to note that Em(en, en) proceeds in a perfectly normal way. Evidently embryos do not fail to complete gastrulation because bottle-cell formation is retarded by this -SH inhibitor.

2,4-Dinitrophenol (DNP)

Gastrulae immersed at Stage 10 in 1.1 × 10^{-4} M DNP at once cease developing (Table I). Yet under these conditions explant systems behave very much as they do in standard solution:

(25) Em(en, en) : DNP, 1.1 × 10^{-4} M; (5) ; 5P; 0N; 0I
(26) St((m, en) : DNP, 1.1 × 10^{-4} M; (8) ; 6P; 0N; 2I
(27) Sp(ec, en) : DNP, 1.1 × 10^{-4} M; (2) ; 2P; 0N; 0I
(28) Fu(m, ec, en) : DNP, 1.1 × 10^{-4} M; (2) ; 2P; 0N; 0I

There were two peculiarities: in DNP, Sp(ec, en) was somewhat less extensive than usual; and there was some notochordal self-differentiation in Type III systems, atypical in systems of this type.

It appears, therefore, that failure of some morphogenetic movement not modelled by our explant systems blocks whole gastrulae exposed to 1.1 × 10^{-4} M DNP. It should not be supposed, however, that Em(en, en) and the others *cannot* be blocked by DNP; for explant systems developing in 1.6 × 10^{-4} M DNP do not exhibit *any* of these movements.

Sodium barbital (Barb)

In solutions made 0.01–0.02 M in sodium barbital, embryos will gastrulate at a normal rate; at these concentrations explant systems, also, behave just as they do in standard solution. But in 0.05 M sodium barbital, gastrulating embryos are greatly retarded (Table I), and explant systems are affected also. Only Fu(m, ec, en) continues to occur, and even this activity is less well-marked than usual:

(29) Em(m, en) : Barb, 0.05 M; (1) ; 0P; 1N; 0I
(30) St(m, en) : Barb, 0.05 M; (1) ; 0P; 1N; 0I
(31) Sp(ec, en) : Barb, 0.05 M; (1) ; 0P; 1N; 0I
(32) Fu(m, ec, en) : Barb, 0.05 M; (1) ; 1P; 0N; 0I

No peculiarities of explant systems in sodium barbital were observed, other than that the endodermal bases were less turgid and more limp than usual.

Urethane (Ur)

Whole gastrulae at Stage 10 will develop with occasional abnormalities following immersion in 0.05 M urethane, while in 0.1 M urethane their development is curtailed almost at once (Table I). But, in environments of both sorts, St(m, en) is selectively inhibited. Data for 0.05 and 0.1 M urethane are lumped together, since there are no results on explant systems that differentiate them:

(33) Em(en, en) : Ur, 0.05, 0.1 M; (4) ; 3P; 0N; 1I
(34) St(m, en) : Ur, 0.05, 0.1 M; (4) ; 0P; 4N; 0I
(35) Sp(ec, en) : Ur, 0.05, 0.1 M; (4) ; 4P; 0N; 0I
(36) Fu(m, ec, en) : Ur, 0.05, 0.1 M; (4) ; 4P; 0N; 0I

Thus, while sodium barbital seems to act according to the classic picture of narcotics as general cell-poisons, urethane does not; and since we are regarding St(m, en) as more than a mere caricature of convergent stretching, it might seem tempting to suppose (from (34) and Table I) that whole gastrulae fail to develop in 0.1 *M* urethane because they cannot therein exhibit convergent stretching. But this would be a highly dubious interpretation, for they will often finish gastrulating in 0.05 *M* urethane, in which St(m, en) is also suppressed.

Anaerobiosis, 95 N_2*: 5* CO_2 *(An)*

Gastrulae made anaerobic at Stage 10 are sometimes prevented thereby from developing much further (Table I); but under similar conditions there will occur all four of the surrogate movements that we have been discussing:

(37) Em(en, en) : An; (3); 3P; 0N; 0I
(38) St(m, en) : An; (2); 2P; 0N; 0I
(39) Sp(ec, en) : An; (2); 2P; 0N; 0I
(40) Fu(m, ec, en) : An; (2); 2P; 0N; 0I

Yet Sp(ec, en) is noticeably less extensive in nitrogen than in air, and it is therefore possible that gastrulae are retarded in anaerobic environments at least partly because in such circumstances epibolic spreading is curtailed.

TOTAL CARBOHYDRATE IN ANAEROBIC EMBRYOS AND EXPLANTS

We turn now to giving an account of some experiments that differ somewhat in technique from the foregoing, but that are relevant to the interpretation of the latter and to the whole question of gastrula metabolism.

The work of Heatley (1935), and especially of Jaeger (1945), has convinced all but the most critical that, in the process of invaginating, gastrular tissues lose about 30 per cent of their glycogen. When Jaeger showed that gastrula explants are not self-differentiating in this respect, she suggested among other things (possibly in response to a hint by Barth, 1942) that invaginated tissues are brought normally into regions under low oxygen-tension, and that mediation by a Pasteur effect might therefore account for their accelerated glycolytic activity. As she pointed out, this hypothesis is susceptible to test, and she announced that experiments to this end were in progress. These were never completed, but preliminary trials failed to support her suggestion (personal communication to the authors). Because of the general interest attached to this question, we have undertaken to extend her preliminary experiments and have confirmed them completely.

TABLE II

Effect of anaerobiosis upon total carbohydrate content of gastrula explants. Numerals following the ± sign indicate standard deviations. P-values calculated by t-test.

µg. total carbohydrate/µg. dry wt. after 24 hours at 12–14° C.

	Anaerobic	Control	P	No. expts.
Dorsal explants	DR: 0.22 ± 0.02	DL: 0.20 ± 0.02	>0.05	3
Ventral explants	VR: 0.21 ± 0.03	VL: 0.20 ± 0.04	>0.05	3

Our results are presented in Table II. They show clearly that, relative to aerobic controls, no significant change occurs in the carbohydrate content of explants, even after 20–21 hours anaerobiosis at 12°–14° C., under which conditions control gastrulae have reached early Stage 12. But if the hypothesis being tested were correct, these systems should have lost about 30 per cent of their glycogen: a carbohydrate change that would have been easily detected (see METHODS). Consequently, the hypothesis is not supported by the evidence, and the reasons for the disappearance of large amounts of glycogen from invaginating gastrular tissues remain quite obscure.

The moral to be drawn is this: the interpretation of explant behavior, metabolic or otherwise, may be complicated by inexplicable responses to isolation.

Summary and General Comments

We have proposed a way to analyze the developmental failure of embryos exposed to unfavorable environmental circumstances; for instance, to anaerobiosis or to metabolic poisons. Our method is based upon the supposition, not clearly warranted by direct observation of delinquent but intact embryos, that under such conditions the component morphogenetic events of embryonic development are selectively retarded in a manner indirectly identifiable by reference to the behavior under similar conditions of explant systems whose overt movements in favorable environments constitute *models* of normal morphogenetic displacements.

In a crude preliminary demonstration of the utility of the method, we have studied three types of explant systems described by Holtfreter. Systems of these types exhibit severally three sorts of movements—called "Em(en, en)" (endodermal embedding in endoderm), "St(m, en)" (mesodermal stretching in endoderm) and "Sp(ec, en)" (ectodermal spreading on endoderm)—that may be regarded with some plausibility as respectively modelling bottle-cell formation, convergent stretching and epibolic expansion. In addition, one such system exhibits a fourth kind of movement—we have called it "Fu(m, ec, en)" (mesodermal fusion with ectoderm on endoderm)—whose instances do not clearly mimic any sort of morphogenetic event occurring in intact gastrulae.

The method seems to work. To the extent that our models are adequate, we have been able to demonstrate some differential effects upon gastrular movements of various inhibitory environmental agents. Our findings may be summarized in the following way:

(1) Surprisingly, 2,4-dinitrophenol (a respiratory stimulant) and 95 N_2 : 5 CO_2 act alike in depressing Sp(ec, en) but not Em(en, en), St(m, en) or Fu(m, en, en). As a tentative guide to discovery, therefore, we might assume that gastrulae fail in dinitrophenol, and are slowed down in N_2 : CO_2, partly because epiboly (but neither bottle-cell formation nor convergent stretching) is suppressed in these circumstances.

(2) Azide, the -SH reagent p-chloromercuribenzoic acid and urethane are inhibitory to both St(m, en) and Sp(ec, en). They can, therefore, be assumed to retard gastrulation movements at least in part because in their presence convergent stretching and epiboly either cannot occur or else are retarded (as Sp(ec, en) by urethane). None of the three inhibitors affects Em(en, en) (and, hence, bottle-

cell formation). Fu(m, en, en) normally occurs in azide and in urethane. Whether it can occur in p-chloromercuribenzoic acid has not been determined by our experiments.

(3) Among the inhibitors that we have studied, sodium barbital is unique in suppressing all three of Em(en,en), St (m,en) and Sp (ec,en), but in allowing Fu(m,ec,en) to occur to some extent. The precise embryological interest of this result is not clear.

Whatever interest this result may have is somewhat lessened by the finding that NaCl in similar concentrations is equally effective.

From an embryological point of view our results are too sketchy, and from the standpoint of biochemistry the exact modes of inhibitor-action are too little understood, to warrant further postulates linking up metabolic events in gastrulating embryos with morphological ones. And, clearly, the method of analysis we have proposed is limited. Before really fruitful information can be obtained with it (fruitful in the sense of suggesting powerful explanatory hypotheses) a careful classification of morphogenetic movements will have to be made, suited to the demands of this type of experiment. Ingenuity will have to be exercised to design and construct refined and more adequate explant models—with due attention paid to unexplained metabolic vagaries of isolated embryonic tissues, as Jaeger's work with glycogen has warned us. And chemically more specific inhibitors whose modes of action are well-understood will have to be employed (metabolite analogs offer some attractive possibilities here). Nevertheless, in theory, there seems to be no reason why metabolic analyses of developmental events cannot be profitably undertaken in the way proposed.

The authors wish to acknowledge more specifically than in the text the stimulus of Professor Holtfreter's careful and beautiful work with explant systems.

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PARTICLE FILTRATION IN SOME ASCIDIANS AND LAMELLIBRANCHS[1]

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Suspension feeders in the sea obtain their food by filtering finely particulate organic matter from the surrounding water. This suspended organic material ranges in size from macroscopic particles down to colloidal dimensions. It is of importance, therefore, to know how effectively filter feeders retain particles of different sizes. Previous investigations on the dependency of particle retention in lamellibranch gill of particle size have given varying results. Galtsoff (1928) reported that the oyster *Crassostrea virginica* effectively filtered diatoms and dinoflagellates, whereas 70–90% of *Bacterium coli* escaped the gill filter. According to Loosanoff and Engle (1947), however, even 60 μ cells of the flagellate *Euglena* could easily pass through the gills of *Crassostrea virginica,* only 15–80% being strained from the water. ZoBell and Landon (1937) stated that the gills of *Mytilus californianus* retain bacteria very efficiently. In *Mytilus edulis* Jørgensen (1949a) found an almost complete retention of particles a few micra in diameter. In order to determine whether a difference exists in the straining efficiency between the oyster and the mussel gills, further investigations were made on *Crassostrea virginica* and *Mytilus edulis.*

In a previous paper (Jørgensen, 1949b) it was stated that the ascidian *Ciona intestinalis* strained particles even more effectively than did *Mytilus.* In this paper are reported attempts to determine the efficiency with which different proteins are adsorbed by the feeding mucus of *Ciona.*

The authors wish to express their sincere thanks to Professors Dennis L. Fox and Norris Rakestraw for the interest they have shown in this work.

TECHNIQUE

The relative retention of particles of various sizes was determined by comparing the rates at which the particles were removed from suspensions. The rate of removal is a function of both the amount of water transported through the animal per unit time and the retentive efficiency. However, it was found that when the animals were adapted to the experimental conditions and were not disturbed, the rate of water transport was rather constant (see Fox, Sverdrup and Cunningham, 1937). In some experiments two types of particles were present in suspension simultaneously. In such experiments, of course, differences in the rates of removal were directly indicative of differences in retention efficiency.

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[1] Contribution from the Scripps Institution of Oceanography, New Series No. 650.

Suspensions were composed of (1) diatoms (*Asterionella japonica* which form colonies about 200 μ in diameter); (2) colloidal graphite (Aquadag A, obtainable from the Acheson Colloids Corporation, Port Huron, Michigan, U. S. A.); (3) blood hemocyanins (from a crab *Loxorhynchus grandis* and from a gastropod, *Haliotis* sp.); and (4) hemoglobin (from man and a fish *Amphisticus argenteus*).

The colloidal graphite forms stable suspensions in distilled water. Suspensions in sea water were prepared from stock suspensions in distilled water. In sea water, the graphite particles coagulate slowly, and in one or a few days all graphite is deposited as macroscopic particles. The coagulation process proceeds very slowly, however, during several hours following the preparation of the suspension. Measurements have been made of particle sizes in freshly prepared and two hour-old suspensions (Fig. 1). In these examples, it is seen that no, or only small, changes occurred in particle size. By far the greater number were 1–2 μ in diameter. In other instances, however, it was observed that the dominating size increased to 2–3 μ during the first half hour. Thereafter practically no further coagulation might occur during the following 5 hours. The suspensions used in the experiments were never more than some 5 hours old.

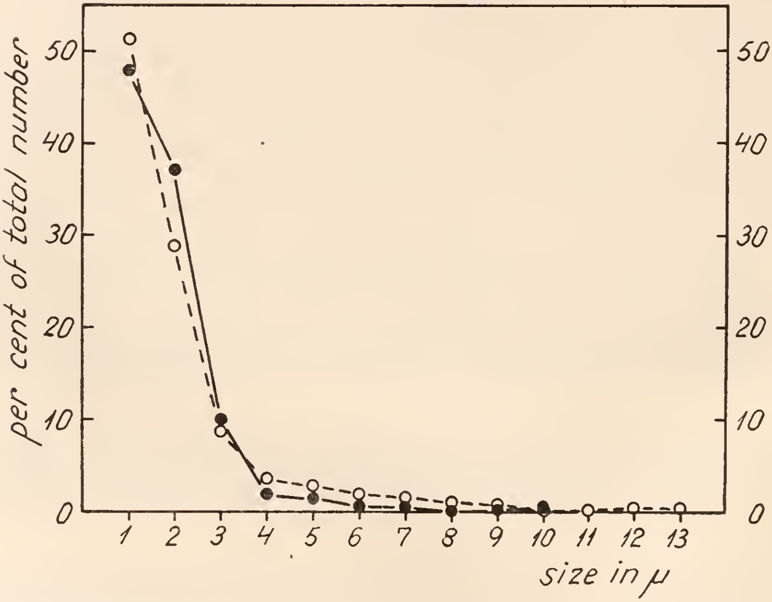

FIGURE 1. Size distribution of graphite particles (Aquadag). ○- - -○, sampled immediately after preparation of the suspension; 505 particles measured. ●—● sampled after 2 hours' agitation with magnetic stirrer; 552 particles measured.

The graphite concentrations were determined colorimetrically by means of a Beckman Model DU Spectrophotometer or a Klett-Summerson photoelectric colorimeter. The initial concentrations of the graphite were 0.2–0.5 mg./liter. It was found that the light absorption, expressed as a function of the concentration of graphite, followed Beer's Law. In experiments wherein the graphite was completely retained in the feeding organs, the proportion of particles removed from the water could therefore directly be determined from the corresponding decrease in light absorption. This, of course, was not strictly permissible when the graphite particles were only incompletely retained in the filters. Under such conditions large particles were removed more effectively than small particles. Since large particles absorb more light than do small particles, the numerical fraction of particles removed was consequently smaller than might correspond to the decrease in light absorption.

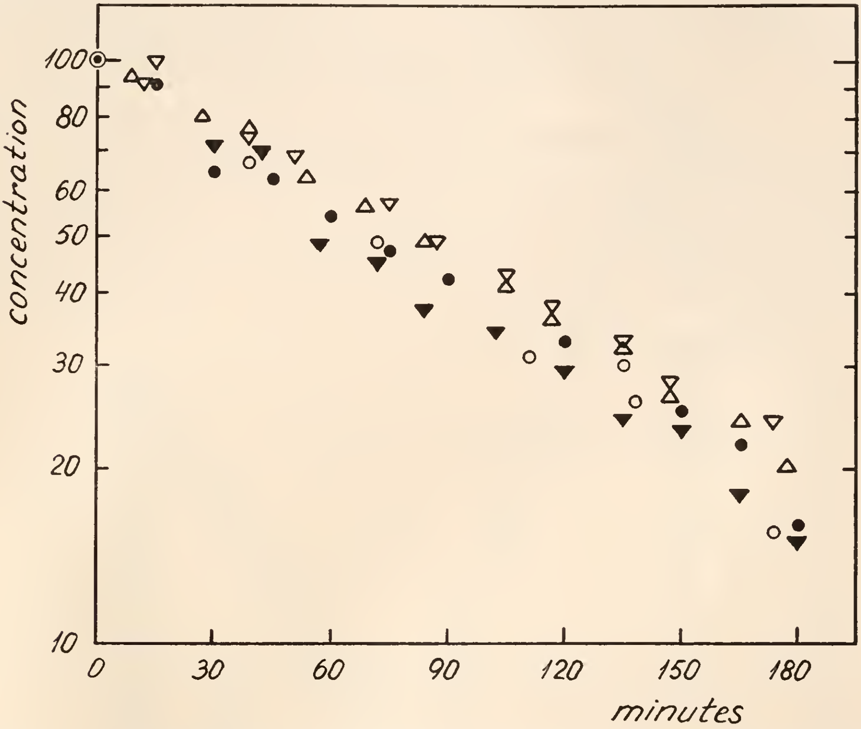

FIGURE 2. *Ciona intestinalis.* Rate of removal of diatoms (● ▼) and colloidal graphite (○ ▽ △). Abscissa: Time in minutes. Ordinate: Concentration in per cent of initial.

The diatoms which were used in the experiments had been cultivated in solutions containing radioactive phosphorus [2] as $P^{32}O_4^{---}$, and had assimilated essentially all the phosphate from the culture media. Samples of the test solution containing the diatoms were evaporated to dryness in planchets under an infrared lamp and assayed for activity under an end-window Geiger-Müller tube with a 1.4 mg./cm.^{2} window thickness. Corrections were made for the small amount of activity leaking into the water from the diatoms.

[2] The radioactive P^{32} used in this investigation was supplied by the Oak Ridge National Laboratory on allocation from the Isotopes Division, U. S. Atomic Energy Commission. See also E. D. Goldberg, T. J. Walker and A. Whisenand (1951).

The hemocyanin and the hemoglobin were assayed spectrophotometrically in 5- or 10-cm. cells. The former suspension was dyed with Evans Blue, T 1824, and excess dye was removed by dialysis against sea water. The measurements were made at 6300 Å. Hemoglobin solutions were obtained by hemolyzing the blood which was eventually diluted to one part per 7000 parts of sea water. The measurements were made at 5700 Å.

Experiments were made on from one to six healthy specimens at a time. Prior to a given experiment, the animals were adapted to the experimental conditions for one to several days. They were kept in 1.0- or 3.5-liter glass vessels. The suspended materials were added cautiously to avoid disturbing the animals. Effective mixing was secured by aeration of the aquaria and aided by the pumping action of the animals. Controls were maintained without animals to ascertain whether any sedimentation or adsorption of the suspended matter occurred during the period of an experiment. In no case did this occur; hence the removal of the suspension was presumed to be due only to the filtering activity of the animals.

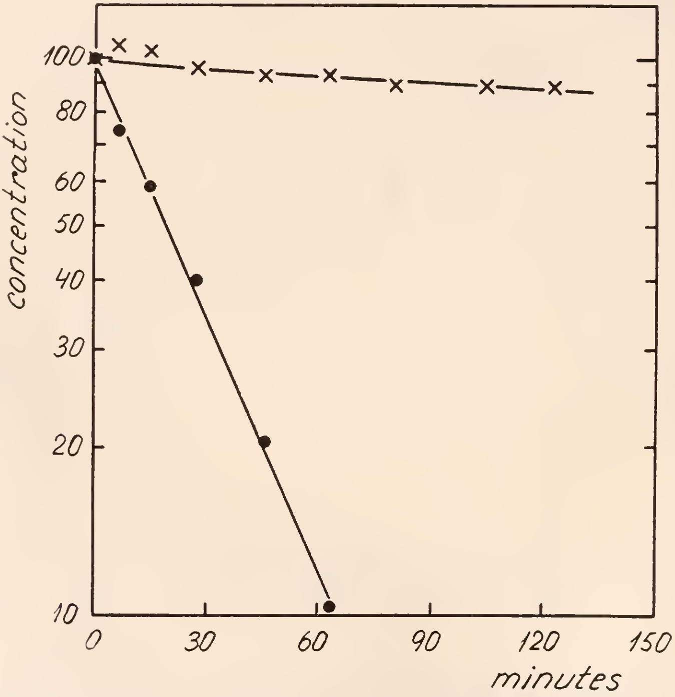

FIGURE 3. *Ciona.* Rate of removal of colloidal graphite (●) and hemoglobin (×) simultaneously present in suspension.

Usually the rate of removal of the different substances was determined by separate experiments. In the case of *Ciona intestinalis,* two suspensions, hemoglobin and graphite, were applied simultaneously. The light absorption was measured at 6500 Å and at 5700 Å. At the former wave-length the extinction is almost exclusively due to the presence of graphite, whereas at the latter wave-length the hemoglobin contributes through an absorption maximum. Graphite was found to absorb equally at both wave-lengths. The specific extinction of graphite, which was computed from the readings at 6500 Å, could therefore be used as a "blank." The specific extinction of hemoglobin was obtained by subtracting the extinction of graphite from the extinction at 5700 Å.

RESULTS

Ciona intestinalis (L.):

Ciona intestinalis specimens were taken both from Mission Bay and from the San Diego Yacht Club Harbor, San Diego, California. Single specimens or clusters of from two to six were used in the experiments.

The relative efficiency with which graphite particles were removed by *Ciona* was determined by comparing the rate of removal of graphite with that of colonies of *Asterionella japonica* which are bigger than the apertures of the branchial basket (Roule, 1884). Hence, all colonies must be filtered from the water passing the pores. Figure 2 shows some typical results of the rate of removal of graphite and the diatom colonies. In these and the following graphs, the logarithm of the

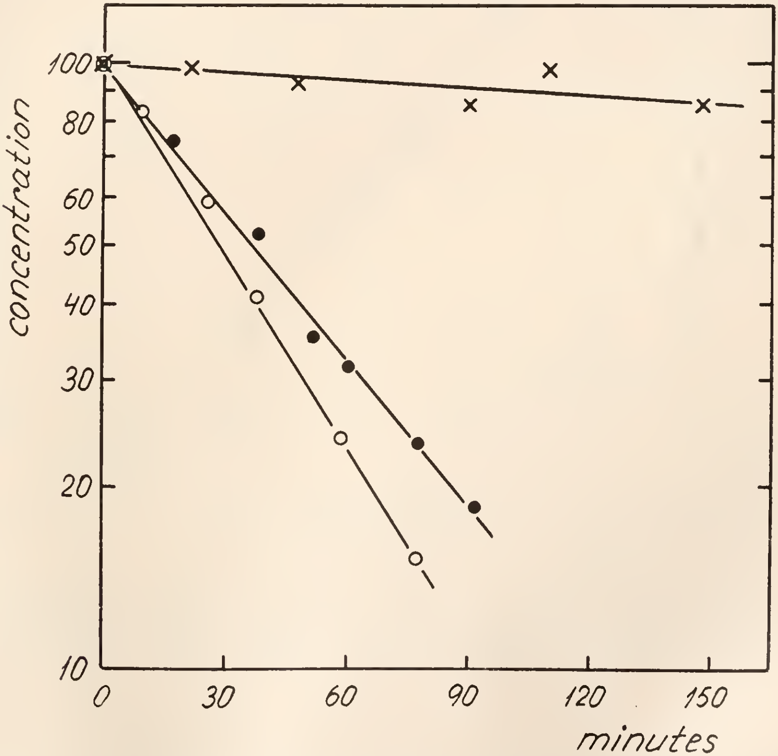

FIGURE 4. *Ciona*. Rate of removal of gastropod (*Haliotis*) hemocyanin (×) and graphite (○ ●).

concentration is plotted against time. The initial concentration is assigned the value of 100. No significant difference was found in the rates of removal of the two materials, even when freshly prepared suspensions of graphite were used. Therefore particles down to 1–2 μ in diameter are effectively removed by *Ciona* from the water passing the branchial basket.

It is also evident from Figure 2 that the curves are nearly straight lines, indicating a constant rate of removal of the suspended material, *i.e.*, a constant rate of water transport throughout the experiments. The relatively constant rate of filtration has always been found in undisturbed, healthy specimens of *Ciona, Mytilus* and *Crassostrea* (see below).

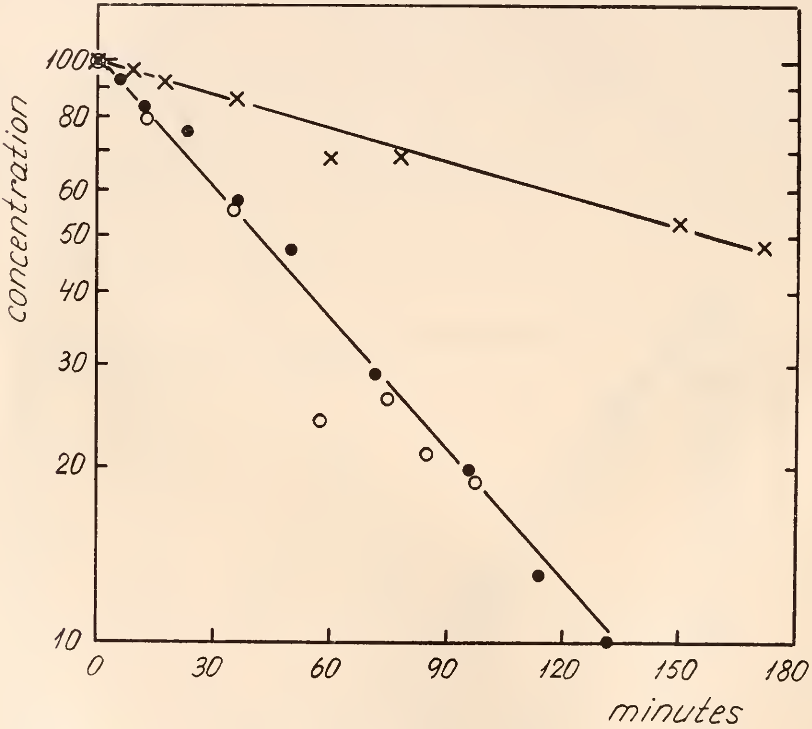

FIGURE 5. *Ciona.* Rate of removal of crab (*Loxorhynchus*) hemocyanin (×) and graphite (○ ●).

In contrast to the effective removal of colloidal graphite, a low degree of retention of the blood proteins was found. In the case of hemoglobin, 90 minutes were required to reduce the hemoglobin concentration by 10%, compared with only three minutes for suspended graphite (Fig. 3). Thus, only a few per cent of the hemoglobin were removed from the water during its passage through the branchial basket. Similar results were obtained with *Haliotis* hemocyanin (Fig. 4), whereas in the only experiment performed with the crab blood, about 50% were extracted in three hours (Fig. 5). More experiments are necessary in order to ascertain whether this difference is significant in the treatment of *Haliotis* and *Loxorhynchus* blood in the feeding organs of *Ciona*.

Mytilus edulis (L.):

Mytilus edulis were taken from the tidal zone off La Jolla, California. All experiments were performed on the same cluster of four specimens, each of which was about 3 cm. in length. Figure 6 shows that the diatoms were removed somewhat more slowly than the graphite. A partial retraction of the mantle edges was observed, however, upon addition of the diatoms, thus suggesting a depression in the activity of the mussels. Later in the experiments, the animals again expanded and this coincided with an increased rate of removal of the diatoms which closely corresponded to the removal rates of graphite. Since the colloidal graphite was not removed at a slower rate than the diatoms, the graphite particles were retained practically completely. At the end of the experiments, the graphite concentrations were reduced to about 10% of the initial amount added without any demonstrable decrease in rate of removal. Hence, *Mytilus* efficiently filtered particles at least down to 1–2 μ.

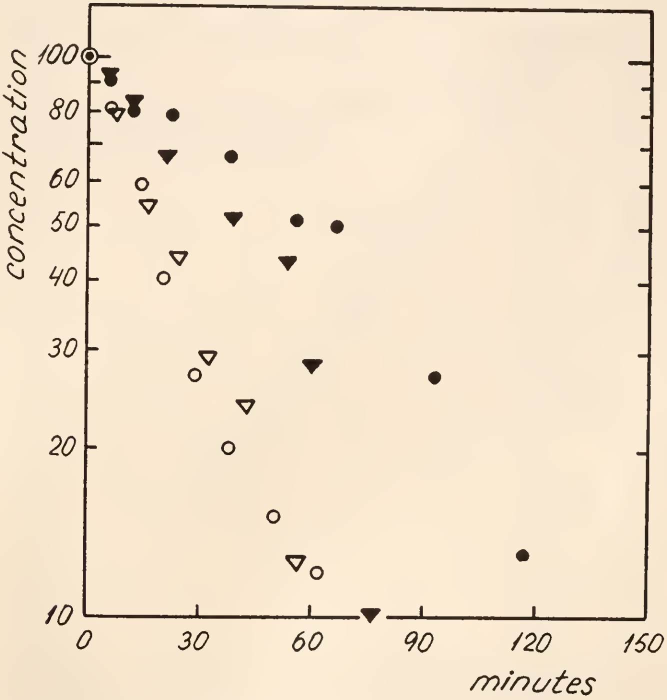

FIGURE 6. *Mytilus edulis.* Rate of removal of diatoms (● ▼) and graphite (○▽).

Crassostrea virginica (Gm.):

The oysters used in the present experiments were kindly placed at the disposal of one of us (C. B. J.) by Dr. P. S. Galtsoff of the U. S. Fish and Wildlife Service, Shellfish Laboratory, Woods Hole, Mass. The animals had been living for about a month in running water in the tanks of the Shellfish Laboratory. The experiments were performed at the Marine Biological Laboratory, Woods Hole. (See also Jørgensen, 1952).

The oysters readily retained all graphite particles in aged suspensions, that is, suspensions more than about one hour old, but removed incompletely the graphite in fresh suspension. Thus, when fresh suspensions of colloidal graphite were offered to the animals, the initial rate of removal was consistently low and decreased with time. Sixteen experiments were carried out, using one oyster at a time. Typical examples are given in Figures 7 and 8. The decreasing removal rates may be due to a preferential retention in the gills of the larger suspended particles causing a decrease in the average size of particles remaining in suspension. Consequently, a less efficient retention in the gill filters resulted. If an aged graphite suspension were exposed to the action of an oyster which was filtering graphite only slowly from a fresh suspension, the rate of removal immediately showed a considerable increase (Fig. 8). In aged graphite suspensions, wherein most particles were

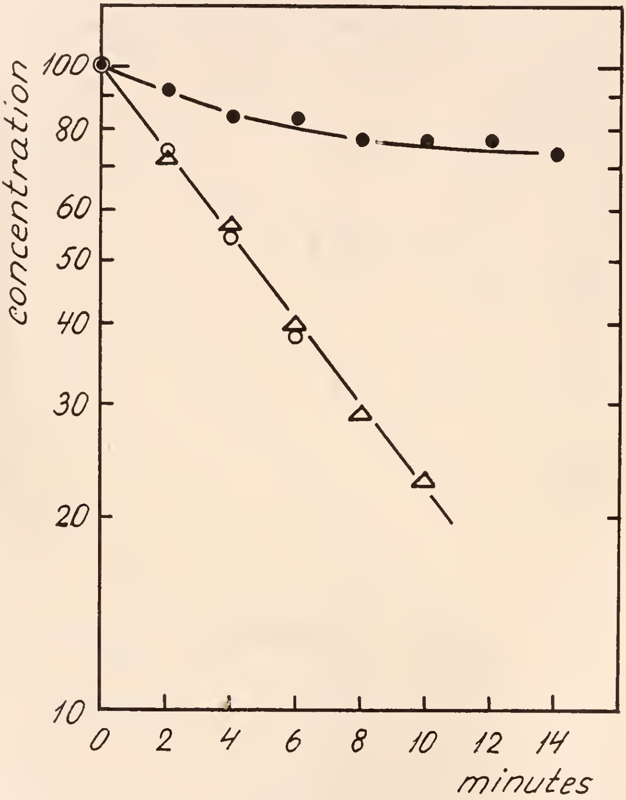

FIGURE 7. *Ostrea virginica.* Rate of removal of graphite from a fresh suspension (●) and from a 4 hours' old suspension (○ △).

about 2–3 μ in diameter, removal rates were high and constant in 26 experiments. Hence, either all the particles or constant fractions were filtered from the water passing the gills. However, it is difficult to understand why a constant fraction of particles of different sizes should be retained when the dominant size was about 2–3 μ but not when the dominant size was about 1–2 μ as in fresh suspensions. Also, the rates of water transport which could be computed from the removal rates of graphite were high in the experiments with aged graphite suspensions, thus indicating a high degree of retention of graphite particles (Jørgensen, 1952). It must therefore be assumed that particles 2–3 μ in diameter and larger were completely retained by the gill filter under the experimental conditions. The graphite in fresh suspension was removed about 5 to 10 times more slowly than the graphite in aged suspensions, showing that fewer than 10–20% of the 1–2 μ particles were retained by the mucus of the oyster gills.

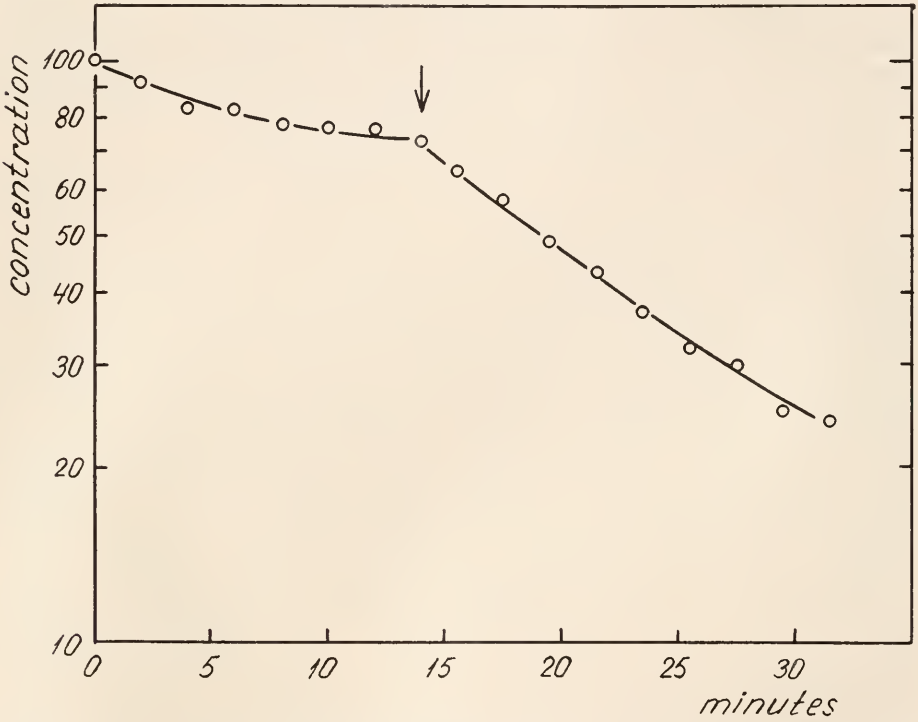

FIGURE 8. *Ostrea.* Rate of removal of graphite from a fresh suspension. At the arrow addition of an aged graphite suspension.

DISCUSSION

MacGinitie (1939) observed that when an ascidian is feeding, a sheet of mucus covers the inside of the branchial basket. This mucus is continuously produced by the endostyle and is carried by cilia to the dorsal food groove which conducts the food-loaded mucus toward the mouth (Orton, 1913; Plough and Jones, 1939).

The presence of the mucus sheet can be nicely visualized in transparent *Ciona intestinalis* by addition of colloidal graphite to the water. When *Ciona* is disturbed no feeding mucus is formed and practically no graphite is retained. In the undisturbed animals, however, blackening of the branchial basket results from addition of graphite suspension. The coloring increases in intensity from the region of the endostyle toward the dorsal groove, as should be expected because the mucus sheet is continually carried dorsally. If excessive amounts of graphite, *e.g.*, several milligrams per liter, are added to the water, the animals presently react by closing the exhalent aperture and contracting the body walls violently, thus ejecting water and mucus incorporated with graphite, through the inhalent aperture. The water present in the chamber between the body walls and the branchial basket is forced through the ostia, carrying with it the mucus which is loaded with graphite. After the contraction, this mucus can be seen floating in the water as large black flakes, whereas no graphite remains on the gills. This clearly shows that the feeding mucus is present as a rather stable, continuously flowing membrane-like structure carried by the transporting cilia of the inside of the branchial basket.

The mucus sheet completely strained 1–2 μ graphite particles from the water. Protein molecules, however, were not effectively retained. More than 90% of the hemoglobin and *Haliotis* hemocyanin passed through the mucus, whereas in a single experiment 25% of crab hemocyanin was retained. In the Gastropoda, the known molecular weights of hemocyanins range from more than one million to close to ten millions, whereas in the Crustacea, the values are lower than one million (Svedberg and Pedersen, 1940). It is thus anomalous that the only experiment made with crab blood proteins showed that the latter were more efficiently retained than were *Haliotis* blood proteins. However, the very big hemocyanins met with in the Gastropoda and other groups are known to dissociate in dilute salt solutions. As a rule it is impossible to predict the molecular size in such dilute solutions, since the dissociation products have been investigated only for a few of the giant hemocyanins. They appear to be stable only around the isoelectric points, *i.e.,* at a pH of about 5. At higher pH values they disintegrate into smaller units which may be ¼ to ⅛ the size of the original molecule (Brohult, 1947; Polson and Wyckoff, 1947).

In addition to observations on ascidians, feeding by mucus sheets has been observed, for instance, in lamellibranchs (MacGinitie, 1941) and in some gastropods (MacGinitie and MacGinitie, 1949; Werner, 1951). In the polychaete *Chaetopterus variopedatus* and the echiuroid worm *Urechis caupo* feeding is performed by means of mucus nets through which the water is pumped (MacGinitie, 1937, 1945). The particle-retaining properties of mucus sheets are apparently different in the different types of filter feeders. MacGinitie (1945) thus found that the mucus comprising the feeding nets of *Urechis* and *Chaetopterus* was unable to retain ovalbumin (molecular weight = 44,000), whereas human serum globulin (molecular weight = 176,000) was partially stopped and *Palinurus* hemocyanin (molecular weight = 450,000) was completely stopped in the mucus nets. These nets are therefore probably less effective than the mucus filter of *Ciona* which only incompletely retained the hemocyanins.

When the gastropod *Crepidula fornicata* is feeding, two sets of mucus filters are formed (Werner, 1951). One is guarding the entrance to the mantle cavity and is mainly removing large particles from the inspired water. Smaller particles pass through, but are strained by the second mucus layer which covers the gills. The dependency of retention on particle size in the two mucus sheets has not been directly measured.

The retention of particulate matter by filter feeding organisms may be a function not only of particle size (reflecting the "porosity of the mucus") but also the charge of the particle relative to the charge of the mucus and possibly of the shape of the particles. Particles with a charge opposite to that of the mucus could be effectively adsorbed. The effect of particle shape and charge on filtering efficiency must await future experimentation.

Lamellibranchia: In *Crassostrea virginica* it was found that graphite particles which were 2–3 μ in diameter were effectively retained by the gills whereas most of the particles about half this size passed through. This is in contrast to the results of Loosanoff and Engle (1947), who found little correlation between particle size and the percentage of particles removed by the gills. The uptake of 5 μ *Chlorella* cells varied from 0 to 92% and of 60 μ *Euglena* cells from 15 to 80%. The concentration of cells was directly measured in the water entering and leaving the oysters. The two bodies of water were separated by a rubber cone enclosing the excurrent side of the oyster.

The properties of the gill filters under these experimental conditions were thus quite different from the properties found in our experiments. The difference in results may be explained by assuming that the oysters were filtering by means of mucus in our experiments but not in those of Loosanoff and Engle. If the mucus sheet were absent, the size of the gill ostia would contribute in determining the degree of retention of particles. Several mechanisms are known which may alter the width of the interfilamental spaces in the gills of Lamellibranchia. Longitudinal muscles in the gill axis and elsewhere can cause a contraction of the entire gill with subsequent narrowing of the slits between the filaments. Vertical muscles in the filaments can produce a shortening and thickening of these with similar effects in the interfilament space. The amount of muscle fibers in the gills varies from species to species (*cf.* Atkins, 1943). If mechanisms of this kind are at work it can be understood how the retention of even large particles can vary from almost complete to practically no retention.

On the other hand, the nearly complete straining, in our experiments, of graphite particles which were only half the size of the smallest cells used by Loosanoff and Engle probably means that mucus sheets, and not the gills proper, performed the straining. Hence, the mucus filtering process in *Crassostrea virginica* effectively retains particles down to about 2–3 μ, only. Similarly, it can be assumed that *Mytilus edulis* was producing mucus sheets in our experiments, and further, that the mucus filter is less porous in *Mytilus* and in *Ostrea* since even 1–2 μ particles were effectively filtered. In previous experiments on *Mytilus edulis* from Plymouth it was observed that the smallest graphite particles generally passed through unadsorbed. This difference between *Mytilus* from California and from Plymouth may be due to differences of specimens from the two localities or due to difference in experimental conditions. From the experiments of Rao (1953) on the variation of pumping rate of *Mytilus californianus* with the latitude, the former explanation appears more reasonable.

Further experiments are needed in order finally to ascertain to what extent formation of mucus filters or adjustment of the size of the interfilamental space without mucus sheet formation are responsible for the effective straining of particles which are only about one or a few microns large.

SUMMARY

1. The efficiency of retention of particles of different sizes has been determined in *Ciona intestinalis, Mytilus edulis* and *Crassostrea virginica* by comparing the rates at which the suspended material was removed from water.

2. The mucus sheet of *Ciona* effectively retained 1–2 μ graphite particles. Only a few per cent of protein molecules such as hemoglobin or gastropod (*Haliotis*) hemocyanin were removed from the water passing through *Ciona*. In one experiment with crab (*Loxorhynchus*) hemocyanin, 25% were retained.

3. *Mytilus* strained practically completely 1–2 μ graphite particles. In *Crassostrea* 2–3 μ particles were effectively filtered from the water, whereas 1–2 μ particles passed through the gills.

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OBSERVATIONS ON MACROPHAGE BEHAVIOR IN THE FIN OF XENOPUS LARVAE

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In a recent study by Niu and Twitty (1950) which dealt with the origin of epidermal melanophores during metamorphosis in the California salamander *Triturus torosus,* the following observations were made. Dermal melanophores which constitute the primary melanophore population during larval stages were observed to undergo autonomous disintegration shortly before metamorphosis. Phagocytic cells which gave the typical staining reactions for macrophages were observed to move into the dermis and ingest the fragments of disintegrating melanophores. Some of these cells, termed "melanophages," were subsequently seen to differentiate into typical melanophores which took their position in the epidermis and were thereafter indistinguishable from other epidermal melanophores in the skin. The conclusion to this paper contained a suggestion which was of particular interest: namely, "that the course of cellular differentiation is susceptible to control or modification in accordance with the specific character of substances transmitted or introduced through the cytoplasm" (p. 647).

The present experiments were undertaken for the purpose of examining this phenomenon in another species in which the details of cellular behavior are particularly favorable for observation. For this purpose the larvae of the clawed African toad, *Xenopus laevis* were used (see Weisz, 1945a, 1945b for normal development). In *Xenopus* larvae, the posterior third of the tail is abundantly supplied with melanophores, but the anterior ⅔ of the ventral fin is entirely free of pigment cells. In this pigment-free region, the fin is very thin and completely transparent so that the details of cell activity can be observed with great clarity.

The animals used in the following study were derived from the *Xenopus* colony maintained at the Zoologisches Institut, Bern. The eggs were obtained by hormonally induced spawning following the injection of estrogenic hormones into the dorsal lymph space of mature male and female animals. The method was that of Gasche (1943) and modified by Andres *et al.* (1948). The eggs were permitted to develop until the feeding stage and thereafter the larvae were fed a suspension of nettle powder as recommended by Gasche (1943, 1944). Larvae ranging in length from 10 to 20 mm. were used in the experiments and all operations and observations were carried out on animals narcotized in one part MS 222 (Sandoz) to 7000 to

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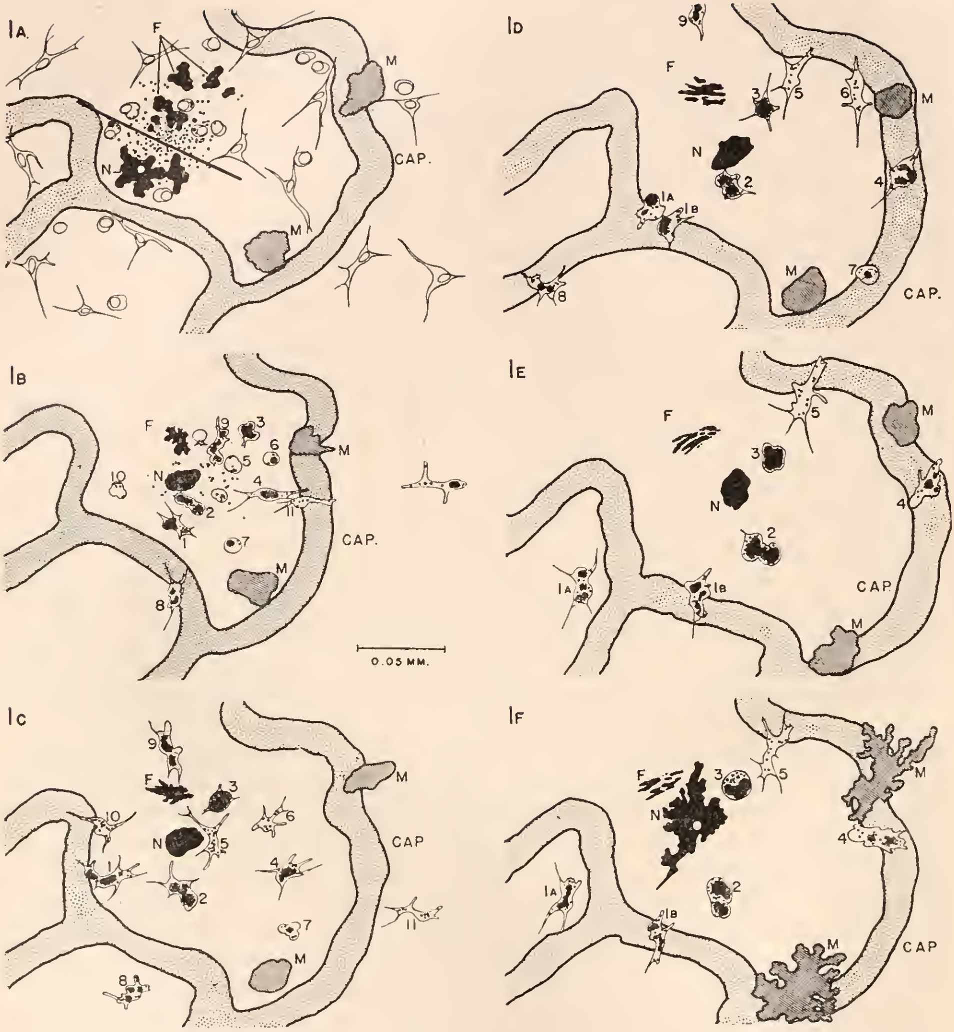
[1] National Science Foundation post-Doctoral Fellow at the Universität Bern, Sept. 1952–Sept. 1953. Now at the University of North Carolina. The writer takes this opportunity to express his deepest appreciation to Professor Fritz Baltzer for his stimulating interest and critical suggestions, to Professor F. E. Lehmann for the living material and numerous creative ideas, and to the staff of the Zoologisches Institut for their cordial hospitality and technical assistance during the tenure of the fellowship.

10,000 parts aquarium water. A special slide was constructed for the observation of the larvae and it served the dual purpose of preventing drying and at the same time elevating the fin so that high power objectives could be brought to bear on the field without endangering the thick head and trunk regions of the body. This slide consisted of a standard 1 × 3 inch slide upon which were mounted two halves of another slide, one on top of the other. They were sealed in place by deKhotinsky cement which is highly water resistant and excellent for this purpose. At one end of the mounted glass pieces was attached a "U"-shaped glass rod, 3 mm. in diameter. The latter provided a small well to hold narcotizing fluid and the head and trunk of the larva. During periods of observation, a small piece of lens paper one cm. square was placed over the head and trunk and capillarity thereafter prevented drying of the delicate epidermis. The tail of the larva extended on the elevated area of the slide and was covered by a standard 22 mm. square cover glass bearing two small drops of bee's wax at the corners placed nearest the body. This supported the cover glass at one end and permitted it to fit the tapered contour of the tail without applying pressure that would interfere with circulation.

The experimental procedures were as follows. Two cubic mm. of the hormone intermedin (2000 units vairon per cc. Intermédin, Choay Lab. Paris) from the intermediate lobe of the pituitary were injected intramuscularly at the base of the tail by means of a fine glass mouth-pipette. This hormone has the well known ability to cause melanophore expansion (Hogben and Slome, 1931; Zondek and Krohn, 1932a, 1932b; Parker, 1940). Two hours after injection, the animals with their melanophores fully expanded were narcotized and one melanophore at the edge of the pigment-free area of the ventral fin was cut into two unequal halves. One part contained the nucleus, and the other consisted of pseudopodial extensions of the cell body (N and F, Fig. 1A–F). All operations were done free-hand at 100 × magnification by means of a finely sharpened steel needle. A small notch was also made at the edge of the fin to mark the general location of the cut melanophore. A camera lucida drawing was made of the region immediately after operation; this figure showed, in addition to the cut cell, the adjacent capillaries, nerves, and melanophores so that there could be no question concerning the position of the cut cell at a later time. These cut cells were observed daily and re-drawn by camera lucida. Most cases were followed for 7 to 10 days, and a few were maintained for as long as 5½ weeks. One representative case history is given in Figures 1A–F.

Shortly after cutting, a few, and frequently many, melanin granules were released from the pigment cell. These granules were freely dispersed in the jelly matrix of the fin in the vicinity of the cell. The amputated pseudopodia tended to contract slightly during the first day following transection, and during the second day phagocytic cells usually completed the process of engulfing all except the largest cell fragments (Figs. 1A, B, C). The latter were frequently observed to remain intact for over a week. The phagocytes which participated in ingestion of melanophore fragments and pigment granules were of two varieties: 1) spherical or oval cells from the blood stream, which entered the field from adjacent capillaries, and 2) stellate mesenchyme cells *in situ* in the matrix of the fin. The former correspond to the "free macrophages" and the latter to the "fixed macrophages" referred to in the literature on the reticulo-endothelial system (see reviews by Levi, 1934; Chevremont, 1942). Preliminary experiments which in part duplicated the results of Vierling


1A
F
N
M
CAP.
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9
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FIGURES 1A-F. *A case history of macrophage ingestion of fragments of a cut melanophore* (camera lucida tracings). *1A,* after 24 hours; *1B,* 2 days; *1C,* 3 days; *1D,* 4 days; *1E,* 5 days, just before injection of intermedin; *1F,* 2 hours after *1E.* Abbreviations; *cap.,* capillary; *F,* non-nucleated melanophore fragment; *N,* nucleated melanophore fragment; *M,* normal melanophores; *1–11,* macrophages with ingested melanophore fragments or melanin granules.

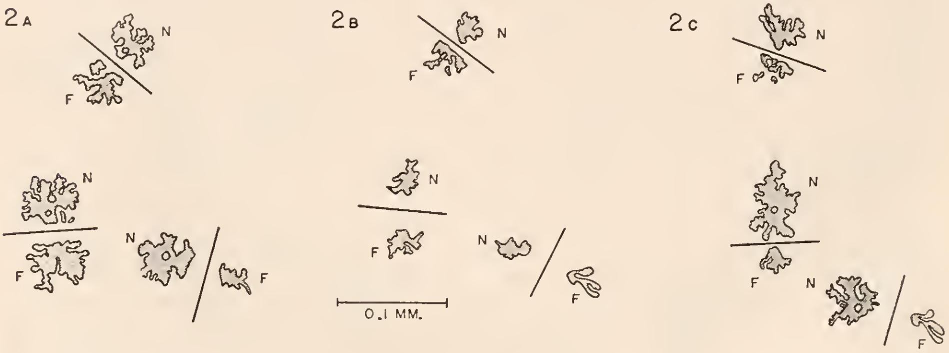
(1926) showed that both of these cell types had a pronounced tendency to accumulate carmine injected into the fin.

The amount of melanin taken up by a single macrophage varied from a few granules (cells 5, 6, 10, 11, Figs. 1B–F), to such great quantities that the cell appeared to be gorged by the ingested particles (cells 1, 2, 3, 4 and 9). In general, those cells which contained only a moderate amount of melanin retained the particles longer than those containing excessive amounts. Some of the cells with fewer than a dozen granules retained these particles for as long as 5 weeks. Macrophages containing large cell fragments usually cast off these particles into the matrix of the fin after 7 to 10 days, or, as was observed in three separate instances, the cells migrated into a capillary and were carried away by the blood stream (cells 7 and 8). The majority of macrophages containing ingested melanin usually remained in the vicinity of the cut melanophore; however, some of these cells showed marked activity (cells 6, 9, 10, and 11) and it was found to be impractical to attempt to follow cells that moved any great distance from the original field.

In none of the cases followed was there any evidence that the macrophages had acquired the ability to synthesize pigment after having phagocytized melanophore fragments. The ingested particles were generally clustered in the interior of the cells in discrete clumps which were quite different in appearance from the arrangement found in normal melanophores. It was not determined whether or not these large aggregations of melanin were contained in vacuoles; however, individual melanin grains appeared to be free in the cytoplasm. Some macrophages with moderate to large amounts of ingested material gave a superficial resemblance to contracted pigment cells (cells 2 and 3) and at first it was thought that these cells might indeed be melanophores in a true sense, since, under usual conditions, the normal melanophores remain in the contracted phase. In order to provide a decisive test to determine whether or not these cells did possess the physiological properties of melanophores, it was decided to see if they would respond in a typical manner to the influence of the hormone intermedin. To this end, four units of the hormone were injected into the base of the tail in the manner already described. Following these injections, not one of the "pseudo-melanophores" responded, whereas host melanophores became expanded maximally, as did the nucleated half of the cut melanophore (see Figs. 1E–F). Since the macrophages gave no response to the hormone, and never gave any indication that they were actively synthesizing melanin, it can be concluded that under the conditions of these experiments, at least, the ingestion of melanin did not stimulate macrophages to become melanophores. In this respect the macrophages of *Xenopus* larvae differed from these observed by Niu and Twitty (1950) in *Triturus torosus*.

It should be borne in mind that the observations of Niu and Twitty were based on normal and not on experimental conditions of behavior. Moreover, the observations on *T. torosus* were made on metamorphosing animals: a time notable for extensive developmental changes which take place under the influence of metamorphosing hormones. The latter fact makes it particularly difficult to compare the results of Niu and Twitty with those on *Xenopus* larvae. Furthermore, it was pointed out by Niu and Twitty that not all macrophages which ingested melanophore fragments in *T. torosus* became converted into melanophores; this particular feature is abundantly confirmed by the present study. Both studies therefore demonstrate that the ingestion of specific types of cellular debris does not play a constant role in determining the fate of the macrophages in question. The only histological transformation of these cells observed in the present experiments was the ready conversion of spherical "free macrophages" from the blood stream into stellate "fixed macrophages" indistinguishable from the mesenchyme cells of the fin matrix (cells 5 and 6). The reciprocal change was not so clearly demonstrable even though some of these stellate cells were observed to migrate into capillaries (cell 8). No clear-cut case was noted in which a mesenchyme cell assumed the typical, pseudopod-free, spherical, character of those cells which originated in the blood stream. A final consideration which should be kept in mind in attempting to compare the results from *T. torosus* and *Xenopus laevis* is the importance of genetic differences which may well play a very significant part in restricting the developmental capacities and behavior properties of macrophages in the two species.

One additional observation on macrophage behavior in *Xenopus* is included at this time even though it is only indirectly related to the foregoing question of macrophage determination following ingestion of cytoplasmic particles. This concerns differences in the response of nucleated and non-nucleated melanophore fragments to the influence of the hormone intermedin. It was noted that within two or three hours after an expanded melanophore was cut, the influence of injected intermedin to a large measure disappeared (Figs. 2A–B). The nucleated half along with the great majority of host melanophores underwent a contraction of granules into a dense mass around the nucleus. The non-nucleated fragments showed less tendency to contract. When intermedin was again injected into these animals two hours after cutting, the nucleated fragments and the normal cells quickly expanded, whereas the non-nucleated parts remained unchanged (Figs. 2B and 2C). These preliminary observations suggest that the cytoplasm of the melanophore requires the presence of the nucleus in order to respond to the expanding stimulus of the hormone intermedin. In any event it would appear that the ability to respond to the hormone is lost by the cytoplasm within the first four hours after separation from the nucleus.



FIGURES 2A–C. The influence of intermedin on nucleated and non-nucleated fragments of melanophores (camera lucida tracings). *2A*, 3 melanophores expanded by an injection of intermedin 2 hours previously and drawn immediately after cutting (*N*, nucleated half; *F*, non-nucleated fragment); *2B*, the same cells 2 hours later, after contraction of melanin has begun and just before a second injection of intermedin; *2C*, the same cells 2 hours after the second injection of intermedin; note that only the nucleated fragments have expanded after cutting.

SUMMARY

1. The hormone intermedin was injected into *Xenopus* larvae to induce pigment cell expansion. In the expanded condition melanophores were cut and macrophage ingestion of melanin granules and cell fragments was observed.

2. Macrophages retained parts of the ingested pigment cells for from one to five weeks. However, in no instance were these macrophages observed to synthesize melanin or to exhibit the physiological characteristics of melanophores as judged by their responsiveness to intermedin.

3. Preliminary observations on the influence of intermedin on nucleated and non-nucleated fragments of melanophores suggest that the cytoplasm of these pigment cells requires the presence of the nucleus in order to respond to the expanding stimulus of this hormone.

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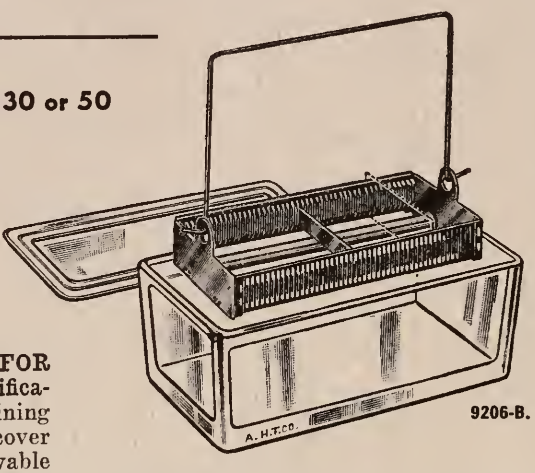

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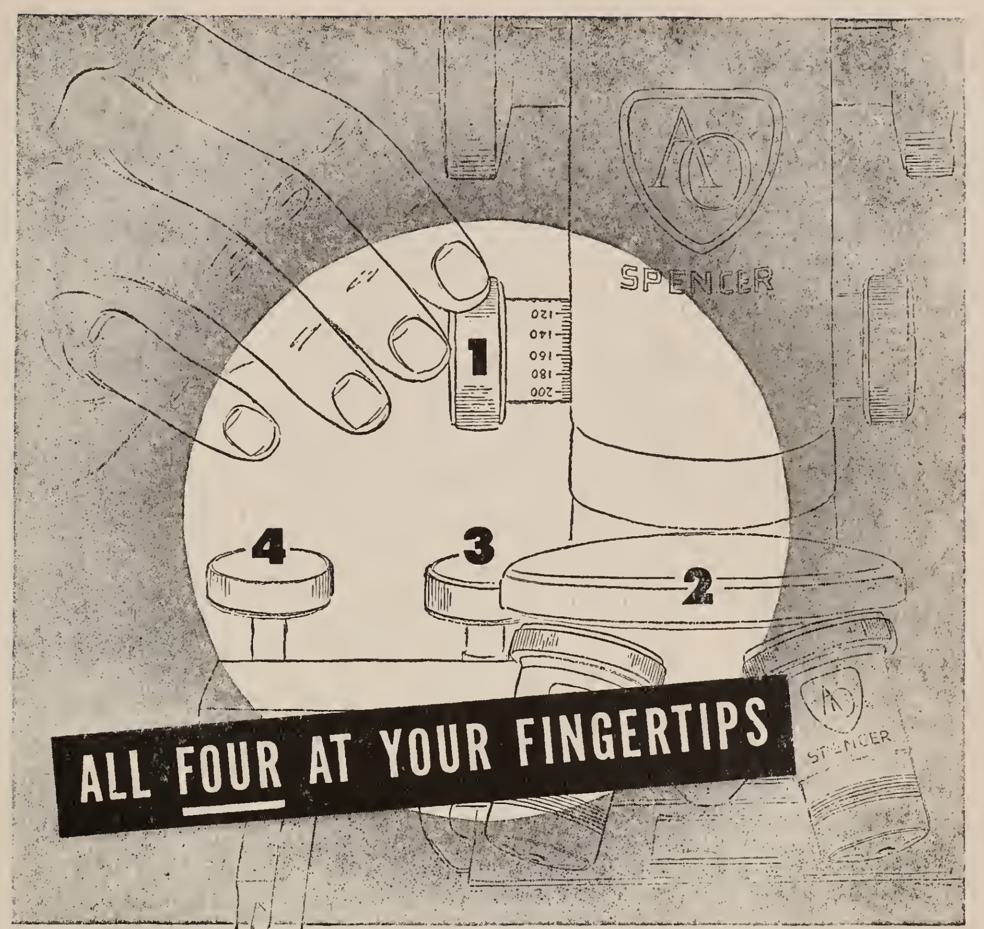

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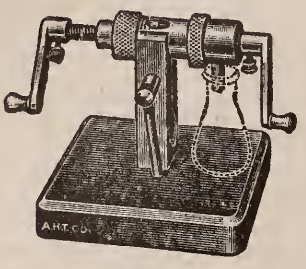

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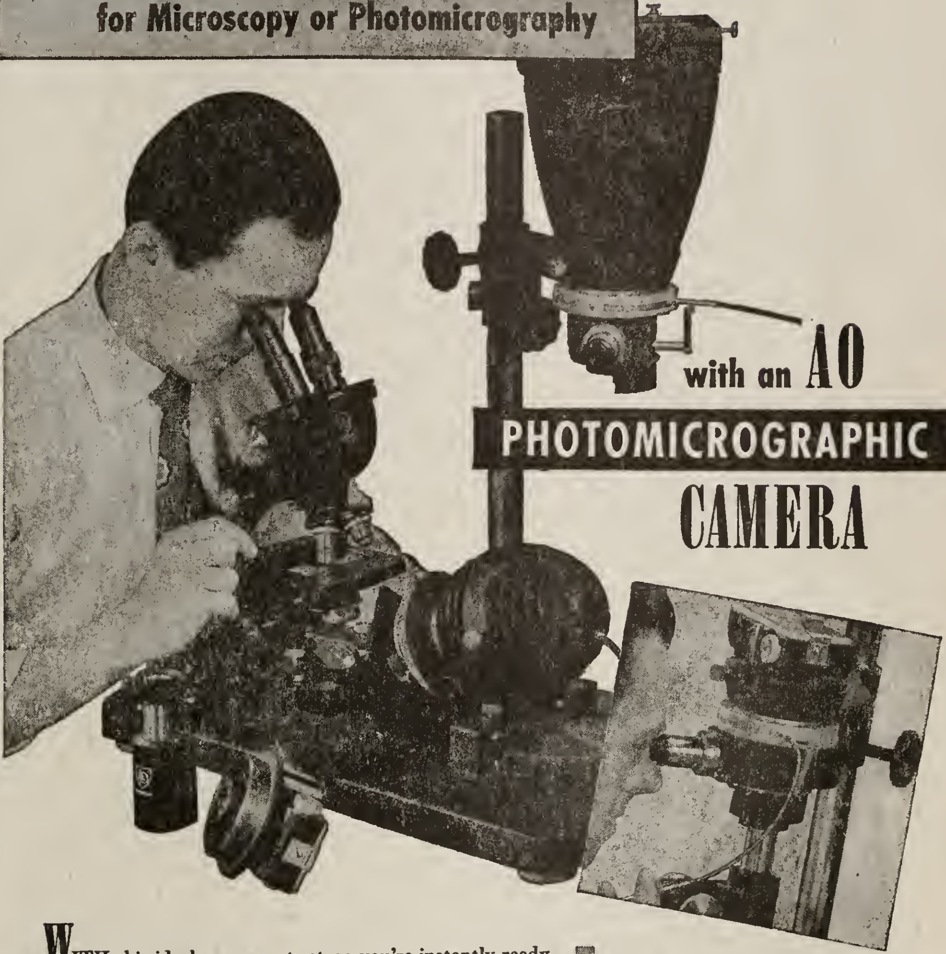

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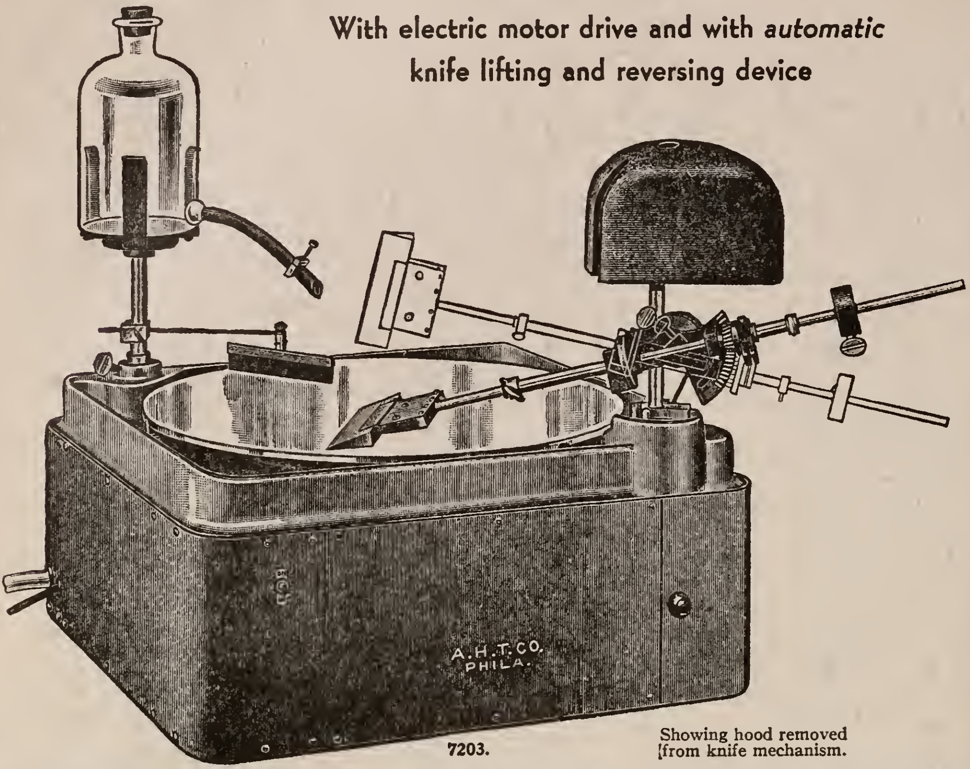

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